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Cu_A and cytochrome c_M from the cyanobacterium *Nostoc* PCC 7120

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Metalloprotein Research Group

Universität für Bodenkultur Wien

Vorstand: Univ. Prof. DI. Dr. Paul Kosma

Betreuer: ao. Univ. Prof. Mag. Dr. Christian Obinger

Eingereicht von

Chantal Lucini

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Abstract

Cyanobacteria were the first oxygenic phototrophic organisms on earth. Gradual modification of the preexisting photosynthetic apparatus could have changed a photosynthetic into a respiratory electron transport chain. Today cyanobacteria are the only organisms capable of both photosynthesis and respiration within a single prokaryotic cell. In addition many species have the capacity to fix nitrogen. In contrast to photosynthesis, the molecular details of cyanobacterial respiration are unknown.

Nostoc sp. PCC 7120 is a filamentous cyanobacterium which can fix nitrogen in terminally differentiated cells called heterocysts. Heterocysts and vegetative cells express two different cytochrome c oxidases, the key enzyme of respiration. In vegetative cells the product of the coxBACI (cox1) gene is found, whereas heterocysts express coxBACII (cox2). Another protein, cytochrome c_M is expressed only under stress conditions and may function as an additional soluble electron donor of cytochrome c oxidase.

In order to understand more about cyanobacterial respiration under stress conditions, a truncated form of subunit II of cox2, SUIIc3 cox2 (i.e. the electron donor binding and electron entry site) and cytochrome c_M were recombinantly expressed in *E. coli* and purified with chromatography.

Recombinant SUIIc3 cox2 lacks copper in its active site and had to be reconstituted. Reconstitution by dialysis with urea was successful but the efficiency of copper incorporation was not satisfying. Expression of the protein as inclusion bodies and reconstitution by dialysis led to higher amount of pure, functional holoprotein, but still not enough to perform comprehensive kinetic measurements.

Cytochrome c_M has been expressed successfully in the presence of several chaperones and purified by a three-step chromatographic purification. However, the small protein was relatively unstable and the yield was too small for kinetic studies.

Zusammenfassung

Cyanobakterien waren die ersten oxygenen phototrophen Organismen dieser Erde. Durch Konversion hat sich später aus dem photosynthetischen Elektronentransport eine Atmungskette entwickelt, sodass Cyanobakterien innerhalb einer (prokaryotischen) Zelle sowohl Atmung als auch Photosynthese betreiben können. Während die molekularen Mechanismen der Photosynthese bereits gut erforscht sind, ist über den respiratorischen Elektronentransport in Cyanobakterien noch wenig bekannt.

Nostoc sp. PCC 7120 ist ein filamentöses Cyanobakterium das Stickstoff in differenzierten Zellen, sog. Heterocysten, fixiert. Heterocysten und vegetative Zellen exprimieren zwei verschiedene Cytochrom *c* Oxidasen, das terminale Schlüsselenzym der Atmung. In vegetativen Zellen wird das Produkt des coxBACI Gen exprimiert, in Heterocysten findet man hingegen das Produkt des coxBACII Gen. Cytochrom c_M ist ein Protein, das nur in Stresssituationen exprimiert wird und vielleicht als zusätzlicher Elektronendonor der Cytochrom c Oxidase dient.

In dieser Arbeit wurden eine verkürzte Form der Untereinheit II der cox2, SUIIc3 cox2 und das Cytochrom c_M in E. coli rekombinant exprimiert und durch Chromatographie gereinigt. Rekombinantes SUIIc3 hat kein Kupfer in seinem aktiven Zentrum und muss rekonstituiert werden. Die Rekonstituierung durch Dialyse mit Harnstoff war erfolgreich, aber die Effizienz des Kupfereinbaus noch zu gering. Durch Expression des Proteins als *Inclusion Bodies* und Rekonstituierung mit Dialyse wurden höhere Mengen an reinem, funktionellem Holoprotein produziert.

Mit Hilfe von Chaperonen konnte intaktes Cytochrom c_M rekombinant exprimiert und nach einer dreistufigen Chromatographie rein dargestellt werden. Allerdings war das Protein instabil und die Menge zu gering.

1. Introduction

1.1 Cyanobacteria

Cyanobacteria are the largest, most diversified, evolutionary most significant and ecologically most successful micro-organisms on our earth. Cyanobacteria were the first and are up to now the only oxygenic phototrophic prokaryotes. Using light as an ubiquitous energy source, liquid water as an ubiquitous electron source ("reductant"), and CO_2 as the most ubiquitous carbon source (together with a few additional minerals) the photo-litho-autotrophic cyanobacteria were indeed, and still are, the nonplus-ultra of bioenergetics [1].

Cyanobacteria are true pace makers of evolution in a geological and a biological sense: they are the most ancient plant-type photosynthetic organisms introducing about 3.2 billion years ago molecular oxygen to an anoxic biosphere [2, 3]. Since they were the first to produce O_2 they may also have been among the first to sense it and utilise it. Gradual modification of the preexisting photosynthetic electron transport and enzyme systems could have changed a photosynthetic into a respiratory chain (conversion hypothesis) [4].

1.1.1 Cyanobacterial photosynthesis and respiration

The most important feature of cyanobacteria is the ability to perform oxygenic (plant type, O_2 releasing) photosynthesis.
An estimated 10^{11} tons of CO_2 per year is converted into biomass by plant-type photosynthesis and the equivalent amount of O_2 is thereby released from water according to Equation (1). Recent estimates assign between 20% and 30% of this worldwide primary productivity to cyanobacteria, in particular to small unicellular marine *Synechococcus* species [5] and likewise unicellular planktonic Prochlorophytes [6, 7]

$6\ CO_2 + 6\ H_2O \leftrightarrows C_6H_{12}O_6 + 6\ O_2$ $\Delta G^{\circ\prime} = \pm 2821.5$ kJ/mol Equation (1)

Nevertheless, the major part of O_2 is released by green plants, where photosynthesis takes place in the chloroplasts, which have their origin in cyanobacteria (endosymbiont hypothesis [8]). The energy conversion apparatus is an integral part of the thylakoid membrane system of these organelles. The first step of photosynthesis is the absorption of light by chlorophyll resulting in electronic excitation, which is passed on from the light harvesting complex to a reaction center where the energy is converted into separation of charge. In essence, light is used to create reducing potential.

During photosynthesis two kinds of light reactions take place: Photosystem I (PSI) generates reducing power employing NADPH and photosystem II (PSII) transfers the electrons of water to a quinone and evolves molecular oxygen at the same time. The electron flow within each PS and between the two photosystems generates a transmembrane proton gradient that drives the synthesis of ATP (photophosphorylation), similar to oxidative phosphorylation.

The principal difference between photophosphorylation and oxidative phosphorylation is the source of highpotential electrons. In oxidative phosphorylation, they come from the oxidation of fuels; in photosynthesis, they are produced by photoexcitation. NADPH and ATP formed by the action of light then reduce CO_2 and convert it by a series of dark reactions (Calvin cycle) which occur in the stroma of chloroplasts, into primarily sucrose and starch (gluconeogenetic pathway) [9].

In cyanobacteria photophosphorylation and oxidative phosphorylation take place within one single prokaryotic cell [10]. A typical cyanobacterium comprises two types of bioenergetically competent membrane systems, viz. the chlorophyll-containing intracytoplasmic or thylakoid membranes (ICM) and the cytoplasmic or plasma membrane (CM). The ICM accommodates the photosynthetic electron transport (PET) as well as the respiratory electron transport (RET) as schematically illustrated in Figure 1.1. They share the plastochinone pool, the cytochrome b_6f complex as well as the soluble electron transfer (ET) proteins cytochrome c_6 and plastocyanin (PC).

However, the cytoplasmic membrane (CM) was shown to contain purely respiratory electron transport chain components and no photosynthetic reaction centres. [10, 11, 12, 13]

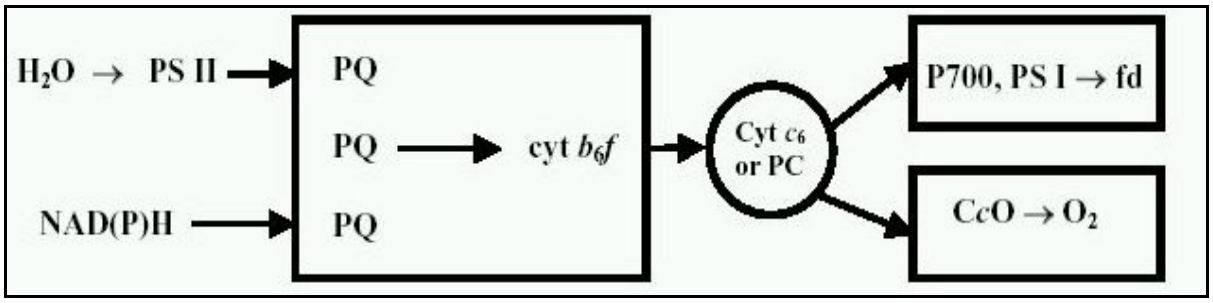


Figure 1.1: *The Dual-function of the photosynthetic and respiratory ET assembly in cyanobacterial ICM. As demonstrated recently [11] either cytochrome* c_6 *or PC is absolutely indispensable for integral electron transport in both photosynthesis and respiration. CcO, cytochrome* c *oxidase; PSI, photosystem I; PSII, photosystem II; fd, ferredoxin; PQ, plastoquinone.*

The rates of cyanobacterial (endogenous) respiration are very low (between 1 and 10% of maximum photosynthetic rates) and most cyanobacteria are, as obligate photo(auto)trophs, unable to sustain efficient growth and proliferation in the dark at the expense of respiration [1, 7]. Thus, cyanobacterial photosynthesis has been receiving much more scientific attention than has respiration. Moreover, most cyanobacteria are obligate photo(auto)trophs unable to sustain efficient growth and proliferation in the dark at the expense of respiration. However, great variations are observed during changes of the physiological state of the cells (growth phase, stress conditions etc.).

In stress conditions, irrespective of the stress type, concentrations and activities of respiratory components are increased, differently in CM and ICM in a species-dependent manner [14, 15]. Furthermore, diazotrophic growth (N_2 fixation in heterocysts or unicells) appears to act as a kind of “physiological stress” anyway imposing an additional energetic burden on the cells which, very typically in these conditions, increase the rate of their (dark) respiratory electron transport (plus oxidative phosphorylation) up to ten-fold; this is also relevant with respect to the respiratory protection of nitrogenase [14]. Considering the fact that it is the primary

impact of most stress types to inactivate photosystem II (PSII) in stressed cyanobacteria (and plants in general) phosphorylation by respiratory electron transport, on the non-photosynthetic CM or on the PSII-inhibited ICM, can temporarily substitute for the lack of an ATP supply from inactivated photophosphorylation and, thus, help the cyanobacteria to survive at least [15].

Given the presence of a photosynthetic electron transport chain in cyanobacterial CM, inherited from anoxygenic ancestors, part of this chain can also serve respiratory purposes in an aerobic environment in accordance with the conversion hypothesis which, in view of the striking similarity of all biological electron transport chains emphasizes the monophyletic origin of biological electron transport [4].

That is why, in all respiring phototrophic prokaryotes, we find a dual photosynthetic and respiratory electron transport chain, which in cyanobacteria, is necessarily confined to ICM. A consequence of the identity of respiratory and photosynthetic electron transport components (in ICM) is the strong functional interaction of the two processes; that is, light inhibits respiration and oxygen inhibits photosythesis under appropriate conditions [18].

1.1.2 *Nostoc* sp. PCC 7120

Nostoc sp. PCC 7120 is a filamentous cyanobacterium that is capable of oxygenic photosynthesis and aerobic nitrogen fixation. Vegetative cells are the sites for photosynthesis, while nitrogen fixation occurs in terminally differentiated cells called heterocysts. In absence of combined nitrogen, approximately every tenth vegetative cell along the filament differentiates into a heterocyst, which provides the anaerobic environment that is required for nitrogenase which reduces N_2 to ammonia. Heterocysts are uniquely effective in protecting this highly O_2-sensitive enzyme from molecular oxygen produced in the neighbouring vegetative cells during oxygenic photosynthesis [19-24].

A semipermeable barrier for penetration of gases is provided by the heterocyst envelope. It consists of an inner glycolipid layer localised just outside of the cell walls'

peptidoglycan layer and an outer polysaccharide layer. Although the van der Waal's radii of nitrogen and oxygen are similar (1.5 Å and 1.4 Å) the barrier by the envelope reduces the permeability of both gases to the extent that the nitrogen that enters still suffices for the needs of the organism for nitrogen, while the oxygen that enters can be reduced to water by respiration [25]. The oxygen-evolving photosystem II complex is inactivated in heterocysts. Therefore they don't carry out oxygenic photosynthesis although they retain the capacity for photosystem-I-dependent photosynthetic reactions. Nitrogen fixation is associated with an increased respiratory activity, reflecting the high energetic costs of the process and also the need to minimize the oxygen level within the heterocyst. Other processes, including peroxidative activities and posttranslational modification of dinitrogenase reductase that limit the damage by oxygen, seem to play a role in the protection mechanism [22, 25-35].

The relationship between heterocysts and vegetative cells is mutualistic. For nitrogen fixation heterocysts require the products of *nif* genes, reductant and ATP. Nitrogen is provided by heterocysts, reductant by vegetative cells. Thus some channels must remain open between the two cell-types. Heterocysts exhibit one to three pores, which provide a route unblocked by the envelope [25].

Nostoc sp. PCC 7120, like other diazotrophs and N_2- fixing cyanobacteria, prefers fixed nitrogen sources. It expresses the system for nitrogen fixation only in the absence of combined nitrogen, since it requires high levels of energy and reductant. Ammonium can independently inhibit the development of both heterocysts and nitrogenase synthesis in heterocystous and also in non heterocystous nitrogen fixing cyanobacteria [19, 25].

1.2 Cytochrome *c* oxidase *(CcO)*

All eukaryotic cells, under heterotrophic growth conditions, synthesized ATP in two ways: by degrading glucose to lactic acid or alcohol via glycolysis, or by oxidative phosphorylation in mitochondria. The latter pathway yields about 15 times more ATP from glucose and is essential for all multicellular organisms [36].

The mitochondrial respiratory chain transfers reducing equivalents from nutrients successively to molecular oxygen, accompanied by storage of the released energy in a proton gradient across the inner mitochondrial membrane via three proton pumping enzyme complexes: (i) NADH-ubiquinone oxidoreductase (complex I), (ii) cytochrome *c* reductase (complex III) and (iii) an aa_3-type cytochrome *c* oxidase, (complex IV). This proton or electrochemical gradient is used by the enzyme ATP synthase for the endergonic synthesis of ATP from ADP and inorganic phosphate [37–39]. The terminal enzyme of the respiratory chain, CcO, reduces molecular oxygen to water without the formation of reactive oxygen species (ROS).

Terminal respiratory oxidases can best be functionally classified as a large superfamiliy of haem-copper oxidases [40, 41] and non-copper or *bd*-type and (certain) *o*-type oxidases [42, 43]. A unifying property of the former is electrogenic proton translocation through the membrane-bound oxidase [44]. On the ground of amino acid sequence similarities and the specific electron donor, *viz.* cytochrome *c* or (*plasto*-)quinol, respectively, cyanobacterial haem-copper cytochrome *c* / quinol oxidases have been separated into three groups: (i) aa_3-type cytochrome *c* oxidases, (ii) haem-copper quinol oxidases, and (iii) putative cytochrome *bd* quinol oxidases [45], the latter do not contain copper but two haems *b* and one haem *d*, do not act as proton pumps, and do not show any sequence similarity with either of the two groups of haem-copper oxidases.

Mammalian C*c*O consists of 13 subunits: In addition to the three mitochondrially encoded subunits I-III, which contain the four catalytic redox centers (Cu_A, heme *a*, heme a_3 and Cu_B) [46], ten smaller subunits are nuclear-coded and partly expressed in tissue-specific isoforms [47-50].

The yeast enzyme contains eleven [51] and the enzyme from the bacterium *Paracoccus denitrificans* (*P. den*.) consists of four subunits, three of which correspond to the mitochondria-encoded subunits of the eukaryotic enzyme [52]. It was postulated, that the increasing number of subunits during evolution reflects an increasing regulatory complexity of the enzyme [47-49, 53]. But only within the last

few years could regulatory functions be identified for some nuclear-coded subunits of the mammalian enzyme.

Crystal structures of C*c*O from bovine heart [54 - 56], *Paracoccus denitrificans* [52, 57] and *Rhodobacter sphaeroides* (*Rb. sph.*) [58] have been determined.

The aa_3-type C*c*O is also the terminal respiratory oxidase of many aerobic bacteria with subunit I-III showing strong homology to their mitochondrial counterparts. Bacterial C*c*Os have simpler structures than mammalian C*c*O, resulting in a lower complexity of physical and genetic structure. Therefore bacterial C*c*Os can be easily manipulated with molecular genetic techniques and are very useful model enzymes, which facilitate a rigorous characterization of the enzyme and eventual understanding of the mechanism of C*c*O. For this reason the structure of C*c*O from *Paracoccus denitrificans* will be briefly discussed below.

1.2.1 C*c*O from *Paracoccus denitrificans* as a model for structure-function analysis

The arrangement of the four subunits (SU) of *Paracoccus denitrificans* (*P. den*.) C*c*O in the membrane and the location of the redox centres are schematically illustrated in Figure 1.2. (A) [52, 59].

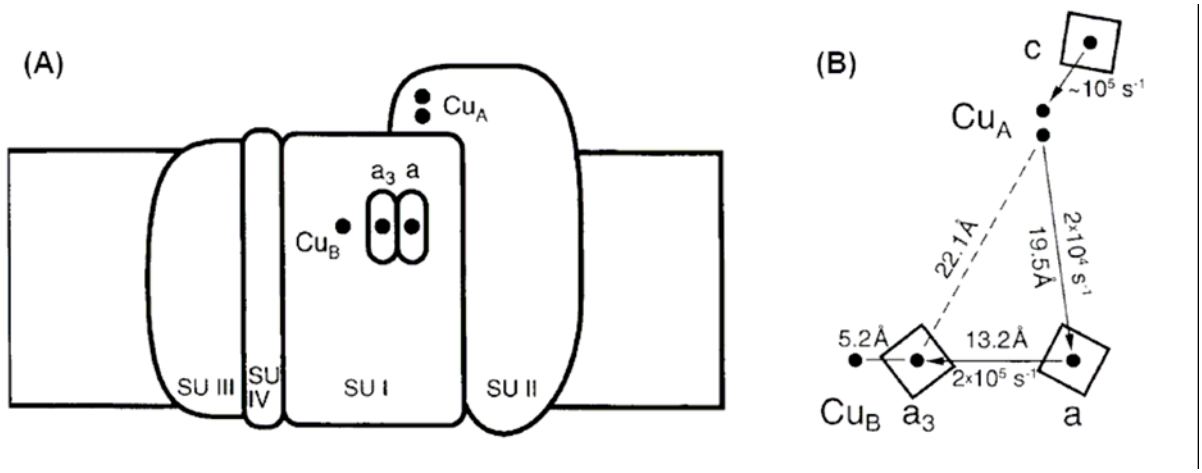


Figure 1.2: (A) *Arrangement of the four protein subunits of C*c*O from P. den. in the membrane and placement of the redox centres.* ***(B)*** *Distances and electron transfer rates between the redox centres in P. den. C*c*O.*

The Cu_A centre, composed of two electronically coupled, mixed-valence copper ions, is located at the hydrophilic domain on the cytosolic side of subunit II. An analysis of site-directed mutants [60] has confirmed the classical view that this centre is indeed the first, and only, acceptor site in the complex. The electron is subsequently transferred from the Cu_A site to heme *a* in subunit I. This low-spin heme is closer to the Cu_A centre than heme a_3, and therefore heme *a* is the kinetically preferred electron acceptor. Both heme planes are oriented perpendicular to the membrane, at an angle of 108° to each other. Their iron centres are 13 Å apart, but their closest edge-to-edge distance is only 5 Å. The high-spin heme a_3 and an electronically coupled Cu_B ion form the binuclear centre of the enzyme [36].

The rate of electron transfer from Cyt *c* in the enzyme-substrate complex is as high as 1×10^5 s^{-1} [61, 62], as illustrated in Figure 1.2. (B). From Cu_A the electron is transferred to cytochrome *a* (cyt *a*) (2×10^4 s^{-1}) and it then equilibrates rapidly (2×10^5 s^{-1}) with cytochrome a_3 (cyt a_3) [63, 64]. These rates have been determined in experiments with the mixed-valence oxidase-CO complex, in which CO dissociation by flash photolysis induces a backflow of electrons from the reduced cyt a_3-Cu_B site to the oxidised cyt *a* and Cu_A site [63-65]. It has been suggested, that the rate of the normal forward ET from cyt *a* to cyt a_3 is limited by proton uptake [63, 66, 67].

1.2.2 Cyanobacterial cytochrome *c* oxidase

In cyanobacteria a mitochondria-like aa_3-type C*c*O appears to be the major terminal respiratory oxidase. Its genes are always clustered in a single operon: coxBAC, encoding subunit II, subunit I and subunit III [34]. Subunit I harbours the heme-copper active site, subunit II contains a copper centre that functions as the primary electron acceptor from cytochrome *c* and/or plastocyanin and subunit III is a membrane protein without redox centers [68-71].

Subunit I

The membrane-embedded subunit I (see Figure 1.3 A) is the largest and most conserved subunit of cytochrome *c* oxidase and contains the active site where

oxygen is reduced. It binds the cofactors of the catalytic centres, the heme-copper binuclear site (high spin heme a_3 and Cu_B) and the low spin heme *a*. In some oxidases subunit I is merged with subunit III or parts of it. It usually contains 12 transmembrane helices with a short loop between helices I and II. Thus the 12 transmembrane helices of cyanobacterial subunit I seem to form an unusually compact confinement (scaffold) for the redox centres. The C-terminal endings of cyanobacterial subunit I is normally about 10 to 20 amino acid residues longer than that of *P. denitrificans*. The transmembrane helices form three arcs within the membrane. Each arc is shaped by four transmembrane helices and together with the last segment of the previous arc pore-like arrangements termed pore A, B, C are formed. The first pore A is filled with mostly conserved aromatic residues, whereas pore B holds the heme a_3-Cu_B binuclear center and pore C contains the low spin heme *a* [17, 72-76].

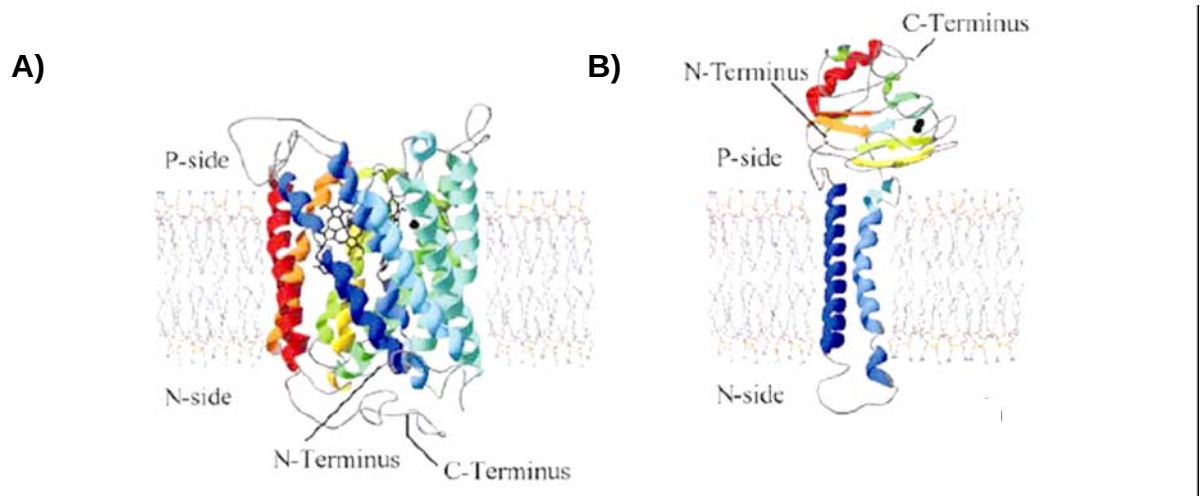


Figure 1.3: *Subunit I A) and II B) of the cytochrome c oxidase in Synechocystis* sp. PCC 6803*. Model building was based on the crystal structure of cytochrome c oxidase from P. denitrificans by using SWISS-Model and the SWISS-PdbViewer (see www. expasy.ch/spdbv/). P-side: periplasmic side, N-side: cytosolic side, copper ions and hemes a and a_3 are depicted in black.*

Subunit II

The Cu_A centre is associated with subunit II (see Figure 1.3.b.). Subunit II is composed of an N-terminal transmembrane helical hairpin followed by a soluble globular domain exposed on the outer membrane surface, whereas latter can be either CM or ICM. The soluble domain of subunit II provides the inner sphere ligands

for the binuclear Cu_A centre. Although the copper site of Cu_A is quite distinct, this domain is related in its protein fold to the cupredoxin fold of simple blue copper proteins [77, 78].

The core of the soluble domain of cyanobacterial heme-copper oxidases compared to *P. denitrificans* cytochrome *c* oxidases shows similar structural elements as a β-barrel formed by 10 β-sheets, but there are significant structural deviations at the N- and C-terminal ends. The fold of the periplasmic domain from *P. denitrificans* is a 10 stranded β-barrel with an overall greek key topology. The loops between strands β3/β4 and β5/β6 contain carboxylic amino acid residues that are conserved among cytochrome *c* oxidases but not among quinol oxidases. These residues have been proposed to form the cytochrome *c* binding site [17, 74].

Cyanobacterial heme-copper-oxidase subunit II displays the typical loop located between the second transmembrane helix and the cupredoxin-like CuA-domain. Both cyanobacterial cytochrome *c* oxidases and quinol oxidases have this unusual insertion, which is not found in *P. denitrificans*, however its length and sequence varies. Sequences of cyanobacterial subunits II include a putative N-terminal signal peptide, which is most likely cleaved off after translocation [17, 78, 79].

Cu_A domain

The Cu_A site is a redox center in cytochrome *c* oxidase and acts as a single electron acceptor and donor. Spectroscopic studies established that the Cu_A-domain is a binuclear, highly delocalised, mixed valence, class IV center, often denoted as [Cu(1.5)-Cu(1.5)]. The single unpaired electron is shared by the two copper ions and is found with equal probability at either metal. Spin delocalisation appears to be a fundamental property of Cu_A centers that distinguishes it from the Fe/S clusters of ferredoxins [72, 80-83].

The key amino acids of the conserved Cu_A site are found near the C-terminal end of subunit II of cytochrome *c* oxidase and are defined by a specific motif, containing one methionine, two histidine and 2 cysteine residues. This part of the protein projects into the periplasmic space and apparently represents an independent folding domain [80, 84].

The Cu_A site is formed by residues from strand β6 and the loop connecting strands β9 and β10 and is basically defined by a Cu_2S_2 core. The two copper atoms, located within bonding distance of each other, are bridged by S_γ atoms of Cys-II-281/249 and Cys-II-285/253 (*Nostoc* sp. PCC 7120 coxBACI/coxBACII numbering) [77, 82]. These four atoms are arranged almost in a single plane. Each copper ion is further coordinated by a histine ligand ($N_{\delta1}$ atoms of His-II-246/214 and His-II-289/257, *Nostoc* sp. PCC 7120 coxBACI/coxBACII numbering).
Axial ligands are the S_δ atom of Met-II-292/260 and the backbone carbonyl atom of Glu-II-283/251 (*Nostoc* sp. PCC 7120 coxBACI/coxBACII numbering). The result is an almost symmetric structure, although the weak axial ligands are different for the individual copper ions. The identity of the ligands is conserved among cytochrome *c* oxidases and also among nitrous oxide reductases that contain a similar Cu_A centre, with the exception of the residue donating the backbone carbonyl oxygen atom [52, 54, 69, 74, 81].

1.2.3 Cytochrome *c* oxidase from *Nostoc* sp. PCC 7120

C*c*O from the filamentous, heterocystous cyanobacterium *Nostoc* sp. PCC 7120 will be briefly described here, as it is the main object of this work.

The genomic sequence of *Nostoc* sp. PCC contains four respiratory operons: coxBACI, coxBACII, coxBACIII, their gene clusters are homologous to coxBAC of *Synechocystis* sp. PCC 6803, and all4023-all4024. The coxBACI (cox1) operon is essential for chemoheterotrophic growth whereas coxBACII (cox2) is the dominating cytochrome *c* oxidase under diazotrophic conditions [21]. coxBACIII (cox3) is designated as a cytochrome *c* oxidase, although its subunit II lacks the Cu_A site. It is similar to *Synechocystis* ARTO [28]. The all4023-all4024 sequences encode a two subunit operon defined as a cytochrome D ubiquinol oxidase [20, 85, 86].

Analysis of coxBACI and coxBACII and the comparison of the amino acid sequence of *Nostoc* sp. PCC 7120 with *P. denitrificans* showed that both encode proteins containing conserved residues known to be required for cytochrome *c* oxidase function. Subunit II of both operons possesses the conserved Cu_A motif, a distinctive

feature of cytochrome *c* oxidases and subunit I from coxAII and coxAI contains three conserved motifs: binding sites for the heme *a* and a_3 and a Cu_B site. [20].

CoxBACI, which appears to be the ortholog of coxBACI operon of *Anabaena variabilis* [86], is constitutively expressed in all vegetative cells with regard to the nitrogen source. No indication of expression in mature heterocysts was obtained so far. Under conditions of nitrogen deprivation every tenth cell differentiates into a heterocyst. During the process of heterocyst differentiation the expression of coxBACII operon is specifically upregulated in heterocysts and proheterocysts. The pattern of expression of the coxBACIII gene cluster is parallel with that observed for coxBACII. Both coxBACII and coxBACIII are bioenergetically active as they contribute to respiration in heterocysts, thus they are essential for nitrogenase activity and diazotrophic growth. Mutants of *Nostoc* sp. PCC 7120 in which either coxBACII or coxBACIII was deleted were still able to grow diazotrophically, whereas deletion of both resulted in almost zero growth under nitrogen-fixing conditions. [20, 28, 34].

1.3 The small soluble electron donors in photosynthesis and respiration: cytochrome c_6 and plastocyanin

Cytochrome c_6 (Cyt c_6) functions as an electron carrier between the cytochrome b_6f complex and either photosystem I [87] or C*c*O [88] in cyanobacteria and in several algae. In the presence of copper plastocyanin (PC) serves as a functionally equivalent electron carrier [89, 90]. In species which can synthesize both Cyt c_6 and PC the expression levels of the corresponding genes are controlled by the copper concentration in the growth medium [91, 92].

During the course of evolution PC seems to have been able to replace Cyt c_6 as PC is the only electron donor in the photosynthesis of higher plants [87]. Nevertheless, database analysis has revealed a gene for Cyt c_6 in higher plants, but significant differences to cyanobacterial or algal Cyts c_6 suggest a modified function [93, 94]. Despite inherent differences Cyt c_6 and the copper protein PC have to share

common physicochemical and structural features in order to accomplish equivalent redox reactions with the same reaction partners.

The total genomic sequence of *Nostoc* sp. PCC 7120 has revealed the presence of three copies genes for Cyt. c_6 [85]. No data are available about the function of these proteins, but one might speculate that the multitude of genes is related to the fact that this strain develops heterocysts upon removal of combined nitrogen [95]. No informations are available about plastocyanin from *Nostoc* sp. PCC 7120. Therefore, to elucidate the structure and function of both proteins, Cyt c_6 and PC from *Synechocystis* sp. PCC 6803 are briefly described in the following chapters.

1.3.1 Cytochrome c_6 from *Synechocystis* sp. PCC 6803

Cyt c_6 from *Synechocystis* sp. PCC 6803 is encoded by the single copy *petJ* gene. The high potential monoheme protein is a member of class I of the cytochrome *c* family, which is mainly characterized by its highly conserved N-terminal heme coordination sequence C-X-X-C-H and a low spin heme with methionine and histidine as axial ligands. The heme prosthetic group is covalently attached to the protein by 2 thioether bonds between the cysteine residues of the heme binding motif and heme rings C and D. It comprises a single protoporphyrin IX with a centrally localized low-spin iron, and the octahedral, six-fold coordination of the iron is completed by histidine and methionine as axial ligands [96].

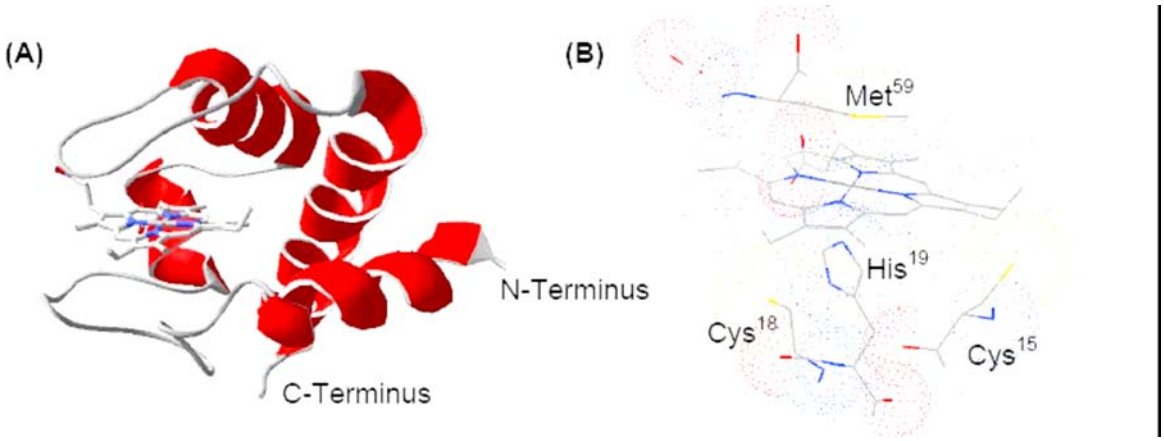


Figure 1.4: (A) *3D model of Cyt c6 from Syn. using SWISS-Model and the SWISSPdbViewer.*

The model building was based on the crystal structure of Cyt c_6 from Porphyra yezoensis (PDB code 1GDV). The four typical α*-helical segments are shown in red, coils in grey. The heme group is shown.* ***(B)*** *Close-up of the heme group with ligands of the Synechocystis Cyt c_6 model.*

Maturation of *c*-type cytochromes involves posttranslational events. These include the targeting of the protein and heme to the correct subcellular compartment, the processing of the targeting sequence, heme attachment and the formation of the covalent linkages. The molecular mechanisms involved are still not quite clear and in discussion.

1.3.2 Plastocyanin from *Synechocystis* PCC 6803

Plastocyanin (PC) is classified as a “small blue” or type I copper protein and in recognition of its redox-function as a cupredoxin. PC forms a 7 stranded β-sandwich and contains one copper atom per molecule.

The Cu atom is coordinated by the side chain of His-40, Cys-84, His-87 and Met-92 (see Figure 1.5) and the Cu-site geometry is best described as “distorted trigonal pyramidal”. The Cu site is located near one end of the molecule. Neither the Cu atom nor its ligands are accessible from the solvent, with the exception of the imidazole ring of the His^{87}. The shortest distance from the Cu atom to the molecular surface, i.e. to the imidazole ring edge of His-87, is about 6 Å [97].
The surface of the protein in the vicinity of the copper site includes a hydrophobic patch. It has been proposed that this patch is the likely site of interaction between plastocyanin and its redox-partner proteins [98].

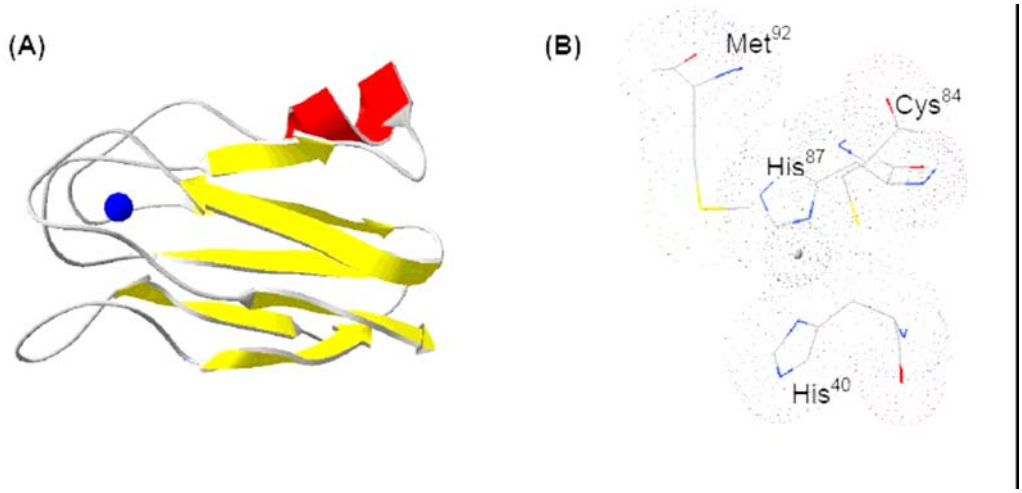


Figure 1.5: **(A)** *3D structure of PC from Syn. (PDB code 1J5C). α-helical segments are shown in red, β-strands are shown in yellow and coils in grey. The copper ion is depicted in blue.* ***(B)*** *Close-up of the PC active site with ligands. The copper ion is drawn as grey sphere.*

1.4 Cytochrome c_M

The total genomic sequence of *Nostoc* sp. PCC 7120 has revealed the presence of a gene coding for cytochrome c_M (Cyt. c_M) [85] but up to now, nothing is known about the protein in this cyanobacterium, but there are some publications about cytochrome c_M in *Synechocystis* sp. PCC 6803.

The gene coding for cytochrome c_M has been found in *Synechocystis* sp. PCC 6803 [99]. It has been detected by Western blotting, but could not be isolated from cells in its mature form, and its physiological function is still unknown [100].

It has been shown that in cells grown under normal conditions, the mRNA level of cytochrome c_M is very low, and mutants lacking Cyt c_M grow normally under photoautotrophic conditions, exhibiting rates of photosynthesis and respiration that are comparable to wild-type cells [99, 101, 103, 104].

Interestingly, under stress conditions (low temperature and high light intensity), when the synthesis of the two soluble metalloproteins Cyt c_6 and plastocyanin (PC) is repressed, Cyt c_M expression drastically increases.

The biological role of this stress-induced balanced regulation of expression of cytochrome c_M versus cytochrome c_6 and plastocyanin is not yet clear.

Replacement of cytochrome c_6 or plastocyanin by cytochrome c_M might be advantageous under conditions of environmental stress, but Cyt c_M may also play another important role as a protective agent against photo-induced and/or oxidative stress [100, 105].

Metzger *et al*. [104] proposed that cytochrome c_M may serve as an alternative carrier to Cyt c_6 or PC in photosynthetic electron transport in *Synechocystis* PCC 6803, analogous to cytochrome c_Y in *Rhodobacter capsulatus* [106]. This suggestion appeared to be supported by the observed stress-induced balanced regulation of expression of cytochrome c_M versus cytochrome c_6 and plastocyanin mentioned above.
However, laser flash-induced kinetic analysis of photosystem I reduction by Cyt c_M, Cyt c_6 and plastocyanin from *Synechocystis*, conducted by Molina-Heredia *et al.*, disproved this hypothesis, as the determined bimolecular rate constant for the overall reaction was up to 100 times lower with (soluble) cytochrome c_M than with Cyt c_6 or plastocyanin [100, 102].

In contrast, Manna and Vermaas [100] have suggested that cytochrome c_M operates in respiratory electron transport, mediating electron transfer from plastocyanin and/or cytochrome c_6 to a caa_3-type cytochrome oxidase (Cyt c_M being linked to C*c*O).
Their suggestion is based on analysis of electron transport in double deletion mutants of *Synechocystis* 6803, and the fact that cytochrome c_M could not be deleted in a photosystem I-less background strain (*i.e.* seems to be necessary for respiratory electron transfer), and also a double-deletion mutant lacking both plastocyanin and cytochrome c_6 could not be obtained (*i.e.* Cyt c_6 and PC are indispensable for respiration as well).

Malakhov *et al*. [99] however have argued that the mature protein in cells is in a soluble form, and the hydrophobic domain is a signal sequence that is cleaved, and it was hypothesised that Cyt c_M could function as an alternative soluble electron donor for C*c*O in respiration.

2. Aim of work

The Metalloprotein Research Group (Biochemistry B) at the Department of Chemistry, BOKU Vienna investigates the structure-function relationship of cyanobacterial *aa*$_3$-type cytochrome *c* oxidase. C*c*O is the key enzyme of cell respiration and cyanobacterial C*c*O is particularly interesting to study as cyanobacteria were the first organisms carrying out oxygenic photosynthesis, producing molecular oxygen in a previously anoxic, O_2-free biosphere. Thus, it seems likely that cyanobacteria were among the first to elaborate a mechanism for aerobic respiration essentially by modifying and adapting a pre-existing photosynthetic electron transport chain [10].

The soluble part of the Cu_A domain of subunit II of C*c*O from *Synechocystis* sp. PCC 6803 was successfully expressed recombinantly in high yield and electron transfer kinetics with the soluble electron donors cytochrome c_6 and plastocyanin were determined [107, 108]. This research revealed that both, the heme- as well as the copper protein can serve as electron donor for the C*c*O. Highly pure recombinant cytochrome c_M was also obtained, however not in high yeld. The bimolecular rate constants for the forward reaction were determined and shown to be comparable with rates between C*c*O and cytochrome c_6 or plastocyanin but the results were not very reliable because of the instability of the reduced cytochrome c_M [109].

The genomic sequence of *Nostoc* sp. PCC 7120contains four respiratory operons. Of particularly interest are the coxBACI (cox1) operon essential for chemoheterotrophic growth and coxBACII (cox2) which is the dominating cytochrome *c* oxidase under diazotrophic conditions [21]. Judith Schachinger successfully overexpressed and purified the soluble Cu_A domain containing part of coxBII (SUIIj3 cox2, amino acid residues 137-327), but could not reconstitute it [110]. A recombinant, purified apoprotein of part of coxBI was also produced by Doris Gusenbauer (March 2007), but the cutting site was set to the amino acid residue 113 (SUIId2 cox1, amino acid residues 113-355), which could interfer with copper binding of the recombinant protein.

Aim of this work was to produce a new recombinant SUII cox2 (amino acid residues 113-327) with the same cutting site as SUIId2 cox1, comprising both the electron donor binding site and the electron entry site but without the two transmembrane helices at the N-terminus. In order to investigate the mode of interaction and kinetics of electron transfer between soluble Cu_A domain and the potential electron donors plastocyanin, cytochrome c_6 and cytochrome c_M, SUII cox2 has to be reconstituted.
Further, it was tried to recombinantly produce and purify the heme protein cytochrome c_M (without the putative signal sequence) in amounts sufficient to perform preliminary kinetic measurements with the Cu_A domain of subunit II of both C*c*Os as reaction. These analyses should shed light on whether Cyt c_M could react with the Cu_A domain of subunit II of both C*c*Os.

3. Material and Methods

3.1 Cloning of recombinant proteins

The soluble part of cytochrome *c* oxidase 2 subunit II (SUIIc3 cox2) and cytochrome c_M of *Nostoc* sp. PCC 7120 were cloned in different *E. coli* strains to allow recombinant expression of the proteins. The insert sequences were amplified by polymerase chain reaction (PCR) and transferred into an expression vector. The plasmids encoding for the proteins were then transformed in electrocompetent cells by electroporation. The proteins were cloned in the following expression system:

Table 3.1: *Recombinant proteins and the used expression systems*

Protein	Number	Amino acid residues	Weight	*E. coli* strain	Expression vector
Cytochrome *c* oxidase 2 subunit II	c3-clone	113-327	22.5 kDa	BL21(DE3)pLysS	pET-3a
Cytochrome c_M	c1-clone	29-105	8.3 kDa	BL21(DE3)Star + pEC68 (heme maturation genes)	pET-27b+

3.1.1 Cloning of SUIIc3 cox2

The insert sequence SUIIc3 cox2 was amplified by PCR and transferred into a pET-3a expression vector.

The pET-3a vector carries an IPTG-inducible T7-promoter sequence, various restriction sites and an ampicillin resistance gene for selection purposes. The plasmid encoding for SUIIc3 cox2 was transformed into electrocompetent *E. coli* BL21(DE3)pLysS cells carrying a pLysS-plasmid coding for T7-lysozym (natural inhibitor of T7-RNA-polymerase). Additionally, pLysS is bearing a chloramphenicol resistance gene. Screening for positive clones was performed by PCR-screening and analysing the expression patterns.

3.1.1.1 Amplification of SUIIc3 cox2 by PCR

❖ **Principles**

PCR is used to amplify target DNA enzymatically. Therefore specific oligonucleotide-primers flanking the target DNA-sequence are designed. The mastermix, consisting of template DNA, DNA-polymerase, oligonucleotide-primers, dNTPs and buffer, runs through several repeating cycles. Each cycle begins with a brief heat treatment to separate the strands (denaturation step), then cooling of the DNA in the presence of a large excess of the two primers allows these primers to hybridize to the complementary sequence in the two DNA strands (annealing step) and finally DNA-synthesis can take place (synthesis step). Because the used primers anneal at high temperature, the annealing gradient extends up to the extension temperature (two-step PCR).

❖ **Protocol**

- 1.5 mL culture of *Nostoc* sp. PCC 7120 was centrifuged 10 minutes at 14000 rpm and the supernatant discarded.
- The mastermix and the negative control were prepared as follows:

Table 3.2: *Components of the mastermix*

	Mastermix	**Negative control**
Primer upstream “lang_DG_het_p1” [10 pmol/µL]	5 µL	5 µL
Primer downstream “lang_DG_het_p2” [10 pmol/µL]	5 µL	5 µL
dNTPs [25 mM]	1 µL	1 µL
5x Phusion HF Buffer	20 µL	20 µL
Template DNA (*Nostoc* sp. PCC 7120)	5 µL	--
Sterile water	63 µL	68 µL

- 99 µL of the mastermix and of the negative were transferred into a PCR-tube.
- The PCR was started using the thermocycler program shown in Table 3.3.
- After 9 minutes 1 µL Phusion DNA Polymerase Hot Start [2 U/µL] was added to the mastermix and the negative control.

Table 3.3: *Thermocycler program*

1		98°C	10 min	
2	denaturation	98°C	10 sec	30 x
3	annealing+ synthesis	72°C	30 sec	
4		72°C	10 min	

❖ **Chemicals and equipment**

Primer: genXpress

N-Terminal: lang_DG_het_p1 [10 pmol/µL]

5' GGGAATTCCATATGAACCAAATGGGAGGATTAGAACCTGGGACTCATCCCC 3'

Nde I Start Insert

C-Terminal: lang_DG_het_p2 [10 pmol/µL]

5' CGCGGATCCCTAATTTACAGACGTTGTATGCAATGTCTCTAAAGTGGCTGC 3'

BamHI Stop Insert

Phusion™ DNA Polymerase Hot Start: Finnzyme (2 U/µL)

5 x Phusion™ HF Buffer: Finnzyme

Template: Pellet of 1.5 mL culture of *Nostoc* sp. PCC 7120

10 mM dNTP Mix: Fermentas

Centrifuge: Sigma 1-15 (Sigma, Germany)

Thermocycler: Techne TC-312

3.1.1.2 Agarose gel electrophoresis

❖ **Principles**

After PCR the amplified insert of SUIIc3 cox2 was analysed by agarose gel electrophoresis performed with E-Gel® stained with SYBR Safe™.

E-Gel® pre-cast agarose gels are self-contained gels that include electrodes packaged inside a dry, disposable, UV-transparent cassette. The E-Gel® agarose gels run in a specially designed device that is a base and power supply combined into one device. The gel contain SYBR Safe™ DNA gel stain.

Agarose gel electrophoresis is used as an analytical or preparative method. In solution DNA is negatively charged and migrates to the anode, when exposed to an

electric field. Due to the sieve-effect of the gel, shorter DNA-fragments migrate faster than longer ones. Therefore DNA is separated according to its size.
SYBR Safe™ DNA gel stain has been specifically developed for reduced mutagenicity, making it safer than ethidium bromide for staining DNA in agarose gels. The detection sensitivity of SYBR Safe™ stain is similar to that of ethidium bromide. DNA bands stained with SYBR Safe™ DNA gel stain can be detected using a standard UV transilluminator.

❖ **Protocol**

- The E-Gel® PowerBase™ v.4 was plugged into an electrical outlet using the adaptor plug on the base.
- The gel was removed from the package and inserted with the comb in place into the base right edge first.
- The gel was pre-run for 2 minutes and the comb removed from the E-Gel® cassette.
- 10 µL of prepared sample were loaded per sample well.
- 10 µL of prepared DNA molecular weight marker (5 µL marker + 5 µL sterile water) were loaded.
- 10 µL of sterile water were loaded into any remaining empty wells.
- Electrophoresis was performed for 15 minutes and the DNA bands were visualised using an UV- transilluminator.

❖ **Equipment**

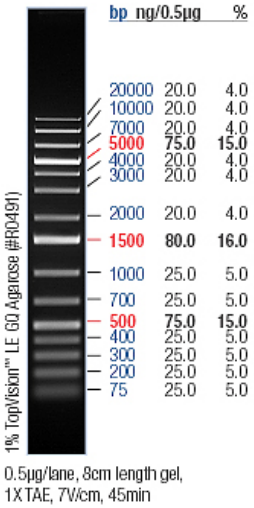
E-Gel® with SYBR-Safe™: Invitrogen

E-Gel® PowerBase™ v.4: Invitrogen

UV-Transilluminator: Gene Flash Syngene Bioimaging

GeneRuler™ 1 kb DNA Ladder: Fermentas

Figure 3.1: *GeneRuler™ 1 kb DNA Ladder*



3.1.1.3 DNA Purification after PCR

❖ **Principles**

For DNA purification of the amplified insert of SUIIc3 cox2 the Gene JET™ Plasmid Mini Prep-Kit was used. Chaotropic salts promote the binding of DNA to the silica-based membrane of the column and denature protein contaminants. Contaminants and salts are removed by subsequent washing with an ethanolic buffer. Purified DNA can be eluted from the glass fibre matrix by sterile water or a buffer of low ionic strength.

❖ **Protocol**

- The GeneJET™ spin column was prepared by placing the column in a collection tube.
- 500 µL Neutralisation solution and 100 µL PCR sample were applied to the column and mixed gently with a pipette. The solution was centrifuged at 14 000 rpm for 60 sec. The flow-through was discarded.
- 500 µL Washing solution (diluted with ethanol prior to first use) were added and centrifuged at 14 000 rpm for 30 sec. The flow-through was discarded.

- The wash procedure was repeated using 500 µL Washing solution.
- The flow-through was discarded and the column centrifuged for an additional minute to remove residual ethanol.
- The GeneJET™ spin column was transferred into a fresh 1.5 mL eppendorf tube. 85 µL sterile water were added to the centre of GeneJET™ spin column membrane to elute the plasmid DNA, incubated for 2 minutes at room temperature and centrifuged at 14 000 rpm for 2 minutes to recover the purified DNA.

❖ **Equipment**

Gene JET^{TM} Plasmid Mini Prep-Kit: Fermentas
Centrifuge: Sigma 1-15 (Sigma, Germany)

3.1.1.4 Preparation of the pET- 3a vector

❖ **Principles**

The pET-3a vector, used for expression of SUIIc3 cox2, carries an IPTG-inducible T7-promoter sequence, various restriction sites and an ampicillin resistance gene for selection purposes.
The Gene JET^{TM} Plasmid Mini Prep-Kit was used for the preparation of the pET-3a vector. Top F10' cells are lysed by an alkaline lysis procedure. The resulting lysate is neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column. Cell debris is pelleted by centrifugation, and the supernatant containing the plasmid DNA is loaded onto the spin column membrane. The adsorbed DNA is washed to remove contaminants, and is then eluted with a small volume of sterile water.

❖ Protocol

- 15 mL of LB_{Amp}-medium were inoculated with Top F10' cells containing pET-3a from a cryo culture and incubated overnight at 37°C and 180 rpm.
- 10 x 1 mL of the culture were transferred into eppendorf tubes and centrifuged 10 minutes at 14 000 rpm. The supernatant was discared.
- The pelleted cells were resuspended in 250 µL of the Resuspension Solution by vortexing.
- 250 µL of the Lysis Solution were added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear.
- 350 µL of the Neutralization Solution were added, mixed immediately by inverting the tube 4-6 times and centrifuged for 5 minutes at 14 000 rpm.
- The supernatant was transferred to the supplied GeneJET™ spin column by carefully pipetting and centrifuged for 1 minute at 14 000 rpm. The flow-through was discared.
- 500 µL Washing solution (diluted with ethanol prior to first use) were added and centrifuged at 14 000 rpm for 30 sec. The flow-through was discarded.
- The wash procedure was repeated using 500 µL Washing solution.
- The flow-through was discarded and the column centrifuged for an additional minute to remove residual ethanol.
- The GeneJET™ spin column was transferred into a fresh 1.5 mL eppendorf tube. 50 µL sterile water were added to the centre of GeneJET™ spin column membrane to elute the plasmid DNA, incubated for 2 minutes at room temperature and centrifuged at 14 000 rpm for 2 minutes to recover the purified DNA.
- To concentrate the plasmid DNA the eluate of two columns was put together and purified with the DNA purification protocol (see chap. 3.1.1.3) until 100 µL concentrated vector-solution remained.
- 5 µL vector were analysed with agarose electrophoresis (see Chapter 3.1.1.2).

❖ **Chemicals and equipment**

Top F10' cells containing pET-3a: Novagene

LB-Medium:

10 g Peptone

10 g NaCl (M_r = 58.44)

5 g Yeast extract

RO-water added to 1000 mL

autoclaved 20 min at 121°C

Gene JET^{TM} Plasmid Mini Prep-Kit: Fermentas

Centrifuge: Sigma 1-15 (Sigma, Germany)

LB-Medium$_{Amp}$:

LB-Medium

100 µg/mL Ampicillin

Ampicillin stock solution:

100 mg/mL RO-water

filter-sterilised and stored ad –20°C

Orbital shaker: Infors HT, Ecotron, Infors AG

Autoclave: Fritz Gössner, Hamburg

3.1.1.5 Digestion of pET- 3a and insert with restriction enzymes

*3.1.1.5.1 Digestion of pET- 3a with Nde*I *and Bam*HI

❖ **Principles**

For insertion of the amplified DNA-fragment into the vector DNA, both have to be digested by the same specific restriction enzymes. The pET-3a vector possesses a multiple cloning site with various restriction sites. The digestion of DNA with the chosen restriction enzymes *Bam*HI and *Nde*I results in linear DNA fragments with so called sticky ends, which can be ligated in a further step (see Chapter 3.1.1.6). Calf intestine alkaline phosphatase (CIP), which is added to the digestion assay of vector DNA, dephosphorylates the sticky ends of plasmid DNA and therefore prevents the ligation of the vector-DNA without an insert.

❖ Protocol

Digestion with *Nde*I:

- The components shown in Table 3.4 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.4: *Components for digestion with Nde*I

84 µL	pET-3a
10 µL	10x buffer 0
6 µL	*Nde*I

The restriction enzyme was added step by step:
1. 3 µL at the beginning
2. 2 µL after 1 hour
3. 1 µL after 1.5 hour

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 82 µL sterile water were used to recover the purified DNA.

Digestion with *Bam*HI:

- The components shown in Table 3.5 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.5: *Components for digestion with Bam*HI

80 µL	pET3a digested with NdeI	
10 µL	10x *Bam*HI-buffer	
6 µL	*Bam*HI	
1 µL	CIP	after 1 h
1 µL	CIP	after 1,5 h

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 50 µL sterile water were used to recover the purified DNA.

*3.1.1.5.2 Digestion of insert with Nde***I** *and Bam***HI**

❖ **Protocol**

Digestion with *Nde*I:

- The components shown in Table 3.6 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.6: *Components for digestion with Nde*I

85 µL	amplified insert of SUIIc3 cox2
10 µL	10x buffer 0
5 µL	*Nde*I
4 µL	sterile water

The restriction enzyme was added step by step:
1. 3 µL at the beginning
2. 2 µL after 1 hour

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 86 µL sterile water were used to recover the purified DNA.

Digestion with *Bam*HI:

- The components shown in Table 3.7 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.7: *Components for digestion with Bam*HI

86 µL	PCR fragment digested with NdeI
10 µL	10x *Bam*HI-buffer
4 µL	*Bam*HI

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 50 µL sterile water were used to recover the purified DNA.

Agarose gel electrophoresis

The digested insert-DNA and pET-3a vector were analysed by agarose gel electrophoresis (See Chapter 3.1.1.2).

❖ **Chemicals and equipment**

***Nde*I:** Fermentas (10 U/µL) ***Bam*HI:** Fermentas(10 U/µL)

10x buffer 0: Fermentas **10x *Bam*HI-Buffer:** Fermentas

Calf intestine alkaline phosphatase: Fermentas

3.1.1.6 Ligation

❖ **Principles**

The amplified and digested insert-DNA is inserted into the vector DNA by ligation. The digestion of double-stranded DNA with the chosen restriction enzymes *Bam*HI and *Nde*I resulted in linear DNA fragments with so called sticky ends, which allow a specific insertion by complementary base pair matching. The enzyme DNA- ligase catalyses the formation of a phosphodiester bridge between the 3' – hydroxyl group of one DNA strand and the 5' – phosphate group of another strand. The molecular concentration ratio between vector and insert DNA should be about 1:10.

❖ **Protocol**

- To verify the molecular concentration ratio 5 µL vector and 5 µL insert were analysed with agarose gel electrophoresis.
- The components shown in Table 3.8 were merged and incubated overnight at 16°C.

Table 3.8: *Components for ligation*

	assay 1	**assay 2**	**negative control**
T4-DNA-Ligase	1 µL	1 µL	1 µL
10x Ligase buffer	2 µL	2 µL	2 µL
Vector DNA	2 µL	2 µL	2 µL
Insert DNA	10 µL	12 µL	---
Sterile water	5 µL	3 µL	15 µL

❖ **Chemicals**

T4-DNA-Ligase: Fermentas
10x Ligase buffer: Fermentas

3.1.1.7 Production of competent *E. coli* BL21(DE3)pLysS cells

❖ **Principles**

Electrocompetent cells are used for electroporation to achieve high transformation efficiency. They are prepared for electroporation under low temperature conditions by transferring them into a medium of low conductivity.

❖ **Protocol**

- 20 mL LB_{Cm}-medium were inoculated with *E. coli* BL21(DE3)pLysS cryo culture and incubated overnight at 37°C and 180 rpm.
- 1 L LB_{Cm}-medium was inoculated with 10 mL of overnight-culture and incubated for 3 hours at 37°C and 180 rpm until an OD_{600} of 0.6 was reached. The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm single use cuvette.
- The culture was incubated in four sterile centrifuge beakers on ice for 30 min and subsequently centrifuged for 6 min at 4°C and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 500 mL of ice cold 1 mM HEPES and centrifuged for 6 min at 4°C and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 250 mL of ice cold 1 mM HEPES and centrifuged for 6 min at 4°C and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 100 mL of ice cold 1 mM HEPES and centrifuged for 6 min at 4°C and 6000 rpm. The supernatant was discarded.

- The pellet was resuspended in 30 mL of ice cold 10% glycerol and centrifuged for 6 min at 4°C and 6000 rpm. The supernatant was discarded.
- The pellet was resuspended in 5 mL of ice cold 10% glycerol.
- 100 µL aliquots of the suspension were transferred into eppendorf tubes and shock frozen in liquid nitrogen and stored at –80°C.
- It is of great importance that besides cooling 1 mM HEPES and 10% also the pellet and collection tubes were cooled on ice.
- The quality of the electrocompetent cells was controlled by transferring 1 mL SOC-medium into a collection tube with an aliquot of electrocompetent cells. Stickiness would be a sign for bad quality.

❖ **Chemicals and equipment**

BL21(DE3)pLysS: Novagene

LB_{Cm}-Medium:

LB-medium

25 µg/mL Chloramphenicol

LB-Medium:

See Chapter 3.1.1.4

Chloramphenicol stock solution:

25 mg/mL 96% Ethanol

stored at –20°C

1 mM HEPES buffer pH 7.0:

autoclaved 20 min at 121°C

10% Glycerol:

autoclaved 20 min at 121°C

Liquid nitrogen

SOC-medium:

5 g NaCl

2.5 g Yeast extract

7.5 g Bacto-agar

RO-water added to 500 mL

autoclaved 20 min at 121°C

Autoclave: Fritz Gössner, Hamburg

Orbital Shaker: Infors HT, Ecotron, Infors AG

UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi

RC-5C Centrifuge: Sorvall Instruments, Du Pont

SLA-Rotor 1500

PHM92 pH meter: Radiometer, Copenhagen

3.1.1.8 Transformation by electroporation

❖ **Principles**

Transformation is the uptake of foreign DNA in a bacterium, thus possibly changing the properties of the organism. Electroporation is an efficient and easy transformation method. Cells are transformed by applying a short high voltage pulse, which creates transient pores and therefore makes the cell membranes permeable for DNA molecules. Length and intensity of the pulse have to be optimised for the different cell types in order to allow pore formation but to prevent lethal cell damage.

❖ **Protocol**

- Aliquots of 1 mL SOC-medium were incubated at 37°C.
- Meanwhile 15 µL of pET-3a/suIIc3 cox2 from ligation assay 1 and 2 and pET-3a from the negative control of the ligation were each transferred to ice cold electroporation cuvettes.
- As negative control 15 µL sterile water and as positive control 3 µL of undigested pET-3a vector after preparation (see Chapter 3.1.1.4) and 12 µL sterile water were used and treated the same way.
- The aliquoted electrocompetent cells were cooled on ice.
- 100 µL of electrocompetent cells were added and mixed thoroughly.
- Electroporation was performed using a Biorad Gene Pulser.
- After electroporation the cells were transferred into the pre-warmed SOC-medium and incubated for 30 min and 37°C.
- 200 µL aliquots of the cells were plated on selective LB_{AmpCm}-agar and incubated overnight at 37°C.

❖ **Chemicals and equipment**

LB-Agar:
5 g Peptone
5 g NaCl
2.5 g Yeast extract
7.5 g Bacto-agar
RO-water added to 500 mL
autoclaved 20 min at 121°C

LB_{AmpCm}-Agar:
LB-agar
100 µg / mL Ampicillin
25 µg / mL Chloramphenicol

SOC-medium:
See Chapter 3.1.1.7

Ampicillin Stock Solution:
See Chapter 3.1.1.4

Chloramphenicol stock solution:
See Chapter 3.1.1.7

Electroporation Cuvettes: 1 mL
Biorad Gene Pulser
Pulse controller: Biorad

3.1.1.9 Screening for a positive clone

3.1.1.9.1 PCR-Screening

❖ **Principles**

PCR is used to screen for successfully transformed clones carrying the recombinant protein, which can be amplified with specific suIIc3 cox2 primers or T7-primers. The first heating step of PCR is used to break down the cells.

❖ Protocol

- A mastermix for 20 assays was prepared (see Table 3.9).

Table 3.9: *Components of Mastermix for PCR screening*

Upstream primer T7 [5 pmol/µL]	9 µL
Downstream primer T7 [5 pmol/µL]	9 µL
dNTPs [25mM]	2.5 µL
10x Buffer + $(NH_4)_2SO_4$	45 µL
Taq DNA Polymerase	4.5 µL
$MgCl_2$	36 µL
Sterile water	194 µL

- Aliquots of 20 µL mastermix were transferred into PCR-tubes.
- In eppendorf cups 50 µL units of sterile water were prepared.
- With an eppendorf pipette one colony was picked and suspended in 50 µL sterile water. 10 µL of the suspension were transferred into a PCR tube and a drop was plated on selective LB_{AmpCm}-agar.
- This procedure was repeated with 12 other colonies. The LB_{AmpCm}-agar-plate was used as masterplate.
- The masterplate was incubated overnight at 37°C and then stored at 4°C.
- 10 µL sterile water were used as negative control and PCR-product (insert after amplification) was used as positive control and was added to the mastermix.
- SUIIc3 cox2 was amplified using the thermocycler program shown in Table 3.10.

Table 3.10: *Thermocycler program*

1	94°C		4 min	
2	94°C	denaturation	40 sec	28 x
3	55°C	anneling	30 sec	
4	72°C	synthesis	1 min	
5	72°C		10 min	

- 10 µL of the amplified samples were analysed with agarose gel electrophoresis.

❖ **Chemicals and equipments**

Primer: Novagene
T7-upstream primer
T7-downstream primer
Taq DNA Polymerase : Fermentas
10x buffer + 200 mM $(NH_4)_2SO_4$: Fermentas
25 mM $MgCl_2$: Fermentas
10 mM dNTP Mix: Fermentas

LB_{AmpCm}-Agar: See Chapter 3.1.1.8
Template: *E. coli* BL21(DE3)pLysS colonies from transformation LB-agar-plate

Thermocycler: Techne TC-312

3.1.1.9.2 Expression-Screening

❖ **Principles**

Small-scale expression experiments are performed to investigate the expression of target protein of successfully transformed cells. The molecular weight of the expressed proteins is checked by SDS-PAGE and compared to the theoretical molecular weight of the SUIIc3 cox2 fragment. Promising cell cultures can be preserved by setting up cryo cultures.

❖ Protocol

- 3 mL LB_{AmpCm}-medium were inoculated with a successfully transformed cell colony and incubated overnight at 37°C and 180 rpm. As positive control 3 mL LB_{AmpCm}-medium were inoculated with a cryo culture of SUIIj3 cox2.
- 20 mL $M9ZB_{AmpCm}$-medium were inoculated with 1 mL of overnight culture and incubated for 4 hours at 37°C and 180 rpm.
- The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm single use cuvette and should be about 1.4.
- The expression was induced with 1 mM IPTG and the suspension was further incubated for 4 hours at 37°C and 180 rpm.
- The culture was transferred in a falcon tube and centrifuged for 15 min at 5000 rpm. The supernatant was discared and the pellet stored overnight at –20°C.
- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14 000 rpm for 3 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.3).

❖ Chemicals and equipment

LB_{AmpCm}-Medium:
LB-medium
100 µg/mL Ampicillin
25 µg/mL Chloramphenicol

LB-Medium:
See Chapter 3.1.1.4

$M9ZB_{AmpCm}$-Medium
800 mL Solution A
100 mL Solution B
100 mL Solution C
1 mL Solution D
1 mL Chloramphenicol [25 mg/mL]
1 mL Ampicillin [100 mg/mL]

Solution A:
10 g Peptone
10 Yeast extract
5 g NaCl (M_r = 58.44)
1 g NH_4Cl (M_r = 53.49)
RO-water added to 800 m
autoclaved 20 min at 121°C

Solution B:
12 g KH_2PO_4 (M_r = 136.1)
24 g Na_2HPO_4 * $7H_2O$ (M_r = 268.1)
RO-water added to 400 mL
autoclaved 20 min at 121°C

Solution C:
16 g Glucose (M_r = 180.16)
RO-water added to 400 mL
autoclaved 20 min at 121°C

Solution D:
12.3 g $MgSO_4 .7H_2O$ (M_r = 246.48)
RO-water added to 50 mL
autoclaved 20 min at 121°C

1 M IPTG stock solution:
238.3 mg IPTG/mL RO-water
filter sterilised
stored at –20°C

Lysis buffer:
50 mM Tris / HCl pH 8.0
2 mM EDTA pH 8.0
0.1 % Triton X-100
RO-water added to 80 mL
1 mM PMSF
0.5 mM DTT

200 mM PMSF:
34.8 mg PMSF in 1 mL Isopropanole

0.5 M DTT:
0.0771 g in 1 mL RO-water

Chloramphenicol stock solution:
See Chapter 3.1.1.7

Ampicillin stock solution:
See Chapter 3.1.1.4

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi

RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500
Centrifuge: Sigma 1-15 (Sigma, Germany)
Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17

3.1.1.10 Set-up of a cryo culture

❖ **Principles**

Cell cultures of clones tested positive in PCR- and expression-screening, can be preserved by setting up cryo cultures using cryo protective agents like glycerol.

❖ **Protocol**

- 3 mL of LB_{AmpCm}-medium were inoculated with a sulIc3 cox2 colony of the masterplate and incubated overnight at 37°C and 180 rpm.
- 900 µL overnight culture and 900 µL 30% glycerol were combined in a cryo vial and mixed gently with a pipette.
- The cryo culture was stored at –80°C.

❖ **Chemicals and equipment**

LB_{AmpCm}-Medium:
See Chapter 3.1.1.9

30% Glycerol:
autoclaved 20 min at 121°C

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
2 mL Cryo Vials: Nalgene

3.1.1.11 Sequencing of plasmid DNA

❖ **Principles**

The sequence of purified plasmid DNA was determined to assure that the plasmid DNA and as a consequence the expressed protein in transformed cells to do not contain mutations. Comparison of the determined sequence with the original one is performed by a software tool. Changes in the nucleotide sequence can cause 'silent mutations', which do not change the amino acid sequence of the protein, or they lead to mutations that alter the amino acid sequence of the encoded protein.

❖ **Protocol**

The DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Purified plasmid DNA (for purification protocol see chapter 3.1.1.2) and primers were provided in the following concentrations:

- 50 µL template (plasmid DNA): 0.2 - 0.5 µg/µL,
- Primers: 5 pmol/µL each

Two primers were used to receive a representative result. Therefore the insert was sequenced starting at the N-terminal- as well as at the C-terminal-end.

❖ **Chemicals and equipment**

T7-Promoter-Primer: Novagene

T7-Terminator-Primer: Novagene

Software: DNAStar SeqMan 4.0

3.1.2 Cloning of cytochrome c_M from *Nostoc* sp. PCC 7120

The insert sequence cytochrome c_M was amplified by PCR and transferred into a pET-27b+ expression vector.

The pET-27b+ vector carries an IPTG-inducible T7-promoter sequence, various restriction sites and an kanamycin resistance gene for selection purposes. This vector is designed for the fusion of target proteins to the pelB signal peptide which facilitates the export into the periplasmic space.

The plasmid encoding for cytochrome c_M was transformed into electro competent *E. coli* BL21(DE3)STAR cells which contain a mutation in the gene encoding RNaseE (*rne*131), which is one of the major sources of mRNA degradation.

The cells used are already carrying the pEC86-plasmid which encodes for the *E. coli* cytochrome *c* maturation genes *ccmABCDEFGH*. The maturation proteins are important for the covalent binding of the heme-group to the apocytochrome. The plasmid also carries a chloramphenicol resistance gene for selection purposes.

Screening for positive clones was performed by PCR-screening and analysing the expression and restriction patterns.

3.1.2.1 Amplification of cytochrome c_M by PCR

❖ **Principles**

See Chapter 3.1.1.1 with the exception that a three-step PCR was performed because the used primers do not anneal at high temperature.

❖ **Protocol**

The same protocol was used as described in Chapter 3.1.1.1, with the exception of the following points:

- N7120_cytcM_CL_pr2 was used as upstream primer and Pr3_cytcM_N7120_Bm was used as downstream primer
- The PCR was performed with the program shown in Table 3.11

Table 3.11: *Thermocycler program*

1		98°C	2 min	
2	denaturation	98°C	10 sec	35 x
3	annealing	60°C	10 sec	
4	synthesis	72°C	10 sec	
5		72°C	10 min	

❖ **Chemicals and equipment**

See Chapter 3.1.1.1 with the exception of the primers:

Primer: genXpress

N-terminal: Pr3_cytcM_N7120_Bm

5’ CATGCCATGGCGATGAAAGGAGACCC 3’

NcoI Start Insert

C-terminal: N7120_cytcM_CL_pr2

5’ GCCCTAGGCTATAAAGTCTCCAAAAAGC 3’

BamHI Stop Insert

3.1.2.2 Agarose gel electrophoresis

See Chapter 3.1.1.2

3.1.2.3 DNA Purification after PCR

See Chapter 3.1.1.3

3.1.2.4 Preparation of the pET- 27b+ vector

See Chapter 3.1.1.4 with the exception that LB_{Kan}-medium was used because pET-27b+ carries a kanamycin resistance gene for selection purposes.

❖ **Chemicals and equipment**

See Chapter 3.1.1.4 with the exception of LB_{Kan}-medium and the kanamycin stock solution.

Kanamycin stock solution:
50 mg/mL RO-water
filter-sterilised and stored ad –20°C

LB_{Kan}-medium:
LB-medium
50 µg/mL Kanamycin

3.1.2.5 Digestion of pET- 27b+ and insert with restriction enzymes

❖ **Principles**

See Chapter 3.1.1.5

*3.1.2.5.1 Digestion of pET- 27b+ with Nco***I** *and Bam***HI**

❖ **Protocol**

Digestion with *Nco*I:

- The components shown in Table 3.12 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table* 3.12: Components for digestion with Nco*I

86 µL	pET-27b+
10 µL	10x buffer Tango
4 µL	*Nco*I

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 82 µL sterile water were used to recover the purified DNA.

Digestion with *Bam*HI:

- The components shown in Table 3.13 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.13: *Components for digestion with Bam*HI

80 µL	pET-27b+ digested with NcoI	
10 µL	10x BamHI-buffer	
6 µL	BamHI	
1 µL	CIP	after 1 h
1 µL	CIP	after 1.5 h

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 50 µL sterile water were used to recover the purified DNA.

*3.1.2.5.2 Digestion of insert with Nco***I** *and Bam***HI**

❖ **Protocol**

Digestion with *Nco*I:

- The components shown in Table 3.14 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.14: *Components for digestion with Nco*I

80 µL	amplified insert of cytochrome c_M
10 µL	10x buffer Tango
4 µL	*Nco*I
6 µL	sterile water

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 86 µL sterile water were used to recover the purified DNA.

Digestion with *Bam*HI:

- The components shown in Table 3.15 were transferred to an eppendorf tube and incubated at 37°C for 2 hours.

Table 3.15: *Components for digestion with Bam*HI

86 µL	amplified insert of cytochrome c_M digested with *Nde*I
10 µL	10x *Bam*HI-buffer
4 µL	*Bam*HI

DNA-purification:

The same protocol was used as described in Chapter 3.1.1.3, with the exception of the following point:

- 30 µL sterile water were used to recover the purified DNA.

Agarose gel electrophoresis

The digested insert-DNA and pET-27b$^+$ vector were analysed by agarose gel electrophoresis (See Chapter 3.1.1.2)

❖ **Chemicals and equipment**

***Nco*I:** Fermentas (10 U/µL) ***Bam*HI:** Fermentas(10 U/µL)

10x buffer Tango: Fermentas **10x *Bam*HI-buffer:** Fermentas

Calf intestine alkaline phosphatase: Fermentas

3.1.2.6 Ligation

❖ **Principles**

See Chapter 3.1.1.6

❖ **Protocol**

- To verify the molecular concentration ratio 5 µL vector and 5 µL insert were analysed with agarose gel electrophoresis
- The components shown in Table 3.16 were merged and incubated overnight at 16°C.

Table 3.16: *Components for ligation*

	assay 1	**assay 2**	**negative control**
T4-DNA-Ligase	1 µL	1 µL	1 µL
10x Ligase buffer	2 µL	2 µL	2 µL
Vector DNA	5 µL	6 µL	5 µL
Insert DNA	7 µL	7 µL	---
Sterile water	5 µL	4 µL	12 µL

❖ **Chemicals and equipment**

See Chapter 3.1.1.6

3.1.2.7 Production of competent *E. coli* BL21(DE3)Star with the pEC86 vector (heme maturation genes)

❖ **Principles**

See Chapter 3.1.1.7

❖ **Protocol**

See Chapter 3.1.1.7 with the exception that LB_{Cm}-medium was used.

❖ **Chemicals and equipment**

See Chapter 3.1.1.7 with the exception of LB_{Cm}-medium and the chloramphenicol stock-solution

LB_{Cm}-Medium:

LB-medium

25 µg/µL Chloramphenicol

Chloramphenicol stock solution:

See Chapter 3.1.1.7

3.1.2.8 Transformation by electroporation

❖ **Principles**

See Chapter 3.1.1.8

❖ **Protocol**

See Chapter 3.1.1.8 with the exception that LB_{KanCm}-agar was used.

❖ **Chemicals and equipment**

See Chapter 3.1.1.8 with the exception of LB_{KanCm}-agar and the kanamycin stock solution.

LB_{KanCm}-Agar:
LB-agar
50 µg/mL Kanamicin
25 µg/mL Chloramphenicol

Kanamycin stock solution
See Chapter 3.1.2.4

3.1.2.9 Screening for a positive clone

3.1.2.9.1 PCR-Screening

❖ **Principles**

See Chapter 3.1.1.9.1

❖ Protocol

- A mastermix for 23 assays was prepared (see Table 3.17).

Table 3.17: *Components of Mastermix for PCR screening*

Upstream primer pr3 [5 pmol/µL]	13.8 µL
Downstream primer pr2 [5 pmol/µL]	13.8 µL
dNTPs [25mM]	3.5 µL
10x Buffer + $(NH_4)_2SO_4$	69 µL
Taq DNA Polymerase	6.9 µL
$MgCl_2$	55 µL
Sterile water	298 µL

- Aliquots of 20 µL mastermix were transferred into PCR-tubes.
- In eppendorf cups 50 µL units of sterile water were prepared.
- With an eppendorf pipette one colony was picked and suspended in 50 µL sterile water. 10 µL of the suspension were transferred into a PCR tube and a drop was plated on selective LB_{KanCm}-agar.
- This procedure was repeated with 19 other colonies. The LB_{KanCm}-agar-plate was used as masterplate.
- The masterplate was incubated overnight at 37°C and then stored at 4°C.
- 10 µL sterile water were used as negative control and PCR-product (insert after amplification) was used as positive control and was added to the mastermix.
- Cytochrome c_M was amplified using the thermocycler program shown in Table 3.18

Table 3.18: *Thermocycler program*

1		94°C	4 min	
2	denaturation	94°C	40 sec	33 x
3	annealing	55°C	30 sec	
4	synthesis	72°C	20 sec	
5		72°C	10 min	

- 10 µL of the amplified samples were analysed with agarose gel ectrophoresis

❖ **Chemicals and equipment**

See Chapter 3.1.1.9.1 with the exception of the primers.

Primers: See Chapter 3.1.2.1

3.1.2.9.2 Expression-Screening

❖ **Principles**

See Chapter 3.1.1.9.2

❖ **Protocol**

- 3 mL LB_{KanCm}-medium were inoculated with successfully transformed cell colonies and incubated overnight at 37°C and 180 rpm.
- 30 mL LB_{KanCm}-medium were inoculated with 300 µL of overnight culture and incubated for 4 hours at 37°C and 180 rpm.
- The optical density OD_{600} was measured photometrically at 600 nm using a 1 cm single use cuvette and should be about 1.4.
- The expression was induced with 1 mM IPTG and 0.2% glycerol. The suspension was further incubated overnight at 20°C and 180 rpm.
- The culture was transferred in a falcon tube and centrifuged for 5 min at 4000 rpm. The supernatant was discarded and the pellet stored overnight at –20°C. If the pellet has a rosa-orange colour, cytochrome c_M is produced by the clone.
- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14 000 rpm for 5 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.6).
- The supernatant was also analysed spectrophotometrically.

❖ **Chemicals and equipment**

LB_{KanCm}-Medium:
LB-medium
50 µg/mL Kanamycin
25 µg/mL Chloramphenicol
LB-Medium: See Chapter 3.1.1.4
Kanamycin stock solution:
See Chapter 3.1.2.4
path)
30% Glycerol
autoclaved 20 min at 121°C

1 M IPTG stock solution:
238.3 mg IPTG / mL RO-water
filter sterilised
stored at –20°C
Lysis buffer: See Chapter 3.1.1.9.2
Chloramphenicol stock solution:
See Chapter 3.1.1.7

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500
Centrifuge: Sigma 1-15 (Sigma, Germany)
Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17
Spectrophotometer: Zeiss Instruments, Diode Array Specord UV-VIS S 10
Cuvettes: Quartz-cuvettes (1 cm light-path)
Software: Aspect plus version 1.5

3.1.2.10 Set-up of a cryo culture

See Chapter 3.1.1.10 with the exception that LB_{KanCm} was used.

3.1.2.11 Sequencing of plasmid DNA

See Chapter 3.1.1.11

3.2 Heterologous overexpression of recombinant proteins

The following clones were used to express the two recombinant proteins. All proteins were expressed using the pET T7 expression systems.

Table 3.19: *Recombinant proteins and the clone used*

Protein	Number	Weight	Amino acid residues
Cytochrome *c* oxidase 2 subunit II	c3-clone	22.5 kDa	113-327
Cytochrome c_M	c1-clone	8.3 kDa	29-105

3.2.1 Expression of SUIIc3 cox2

❖ **Principles**

The soluble part of cytochrome *c* oxidase 2 Subunit II was expressed in the pET-3a plasmid in the *E. coli* strain BL21(DE3)pLysS. The protein was obtained in soluble form in the cytoplasm.

❖ **Protocol**

- 30 mL of LB_{AmpCm}-medium were inoculated with cells from a cryo culture and incubated overnight at 37°C and 180 rpm.
- 12 mL of this overnight culture were taken as inoculum for 1 L $M9ZB_{AmpCm}$-medium, which was divided into two sterile 2 L erlenmeyer flasks.
- The inoculated $M9ZB_{AmpCm}$-medium was incubated at 37°C and 180 rpm until OD_{600} reached 1.4.
- Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG.
- The expression culture was incubated 20 h at 16°C and 180 rpm and subsequently centrifuged for 10 min at 6000 rpm and 25°C.

- The pellet was resuspended in some supernatant and transferred to four falcon tubes.
- The cell suspension was centrifuged for 15 min at 4000 rpm and 25°C. The supernatant was discarded.
- The cell-pellet was stored at –80°C

❖ **Chemicals and equipment**

LB_{AmpCm}-Medium:
See Chapter 3.1.1.1.8
$M9ZB_{AmpCm}$-Medium:
See Chapter 3.1.1.9.2
Solution A:
See Chapter 3.1.1.9.2
Solution B:
See Chapter 3.1.1.9.2
Solution C:
See Chapter 3.1.1.9.2
Solution D:
See Chapter 3.1.1.9.2
Ampicillin stock solution:
See Chapter 3.1.1.4
Chloramphenicol stock solution:
See Chapter 3.1.1.7
1 M IPTG:
See Chapter 3.1.1.9.2

Autoclave: Fritz Gössner, Hamburg
Orbital Shaker: Infors HT, Ecotron, Infors AG
UV-VIS Spectrophotometer: U-1100 Spectrophotometer, Hitachi
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-Rotor 1500

3.2.2 Expression of cytochrome c_M

❖ **Principles**

Cytochrome c_M was expressed in the *E. coli* strain BL21(DE3)Star using the pET-27b+ plasmid. This vector is designed for the fusion of target proteins to the pelB

signal peptide which facilitates the export into the periplasmic space. The periplasm provides oxidising conditions which promote disulfide bond formation and proper folding, which may also enhance the solubility and activity of proteins. In the case of cytochrome c_M the translocation to the periplasmic space is needed for its maturation.

In *E. coli* the covalent heme attachment is achieved by the *ccm* (cytochrome *c* maturation) proteins. The coexpression of the *ccm*A-H gene cluster was achieved by the introduction of the pEC86 plasmid.

To optimise the culture conditions some preliminary tests were performed with varying media, duration of expression, incubation temperatures, IPTG, glycerol and hemin additions.

3.2.2.1 Preliminary tests

- 15 mL of LB_{KanCm}-medium were inoculated with cells from a cryo-culture and incubated overnight at 37°C and 180 rpm.
- 200 µL of this overnight culture were taken as inoculum for 20 mL medium and cultivated under the conditions given in table 3.21.

Table 3.20: *Pre-Test Culture Conditions for Cytochrome c_M expression (in yellow the standard protocol)*

Medium	Temperature	Duration	Glycerol Addition	IPTG Addition	Hemin addition
$M9ZB_{KanCm}$	16 °C	24 h		1 mM	
$M9ZB_{KanCm}$	20 °C	24 h		1 mM	
$M9ZB_{KanCm}$	30 °C	10 h		1 mM	
$M9ZB_{KanCm}$	37 °C	8 h		1 mM	
$M9ZB_{KanCm}$	16 °C	24 h		0.2 mM	
$M9ZB_{KanCm}$	16 °C	24 h		0.5 mM	
$M9ZB_{KanCm}$	16 °C	24 h		1 mM	50 mg/ml
$M9ZB_{KanCm}$	30 °C	10 h		0.2 mM	
$M9ZB_{KanCm}$	30 °C	10 h		0.5 mM	
$M9ZB_{KanCm}$	30 °C	10 h		1 mM	50 mg/ml
LB_{KanCm}	16 °C	24 h	0,2%	1 mM	
LB_{KanCm}	20 °C	24 h	0,2%	1 mM	
LB_{KanCm}	30 °C	10 h	0,2%	1 mM	
LB_{KanCm}	37 °C	8 h	0,2%	1 mM	

- After the indicated duration, cultures were centrifuged 15 min at 4000 rpm. Cell pellets that exhibited a reddish colour were stored at –80°C.

- The pellet was resuspended in 4 mL lysis buffer and sonicated on ice (3 x 40 sec of short pulses). 1 mL was transferred to an eppendorf cup and centrifuged at 14 000 rpm for 5 min.
- The supernatant and the pellet, resuspended in 400 µL RO-water, were analysed by SDS-PAGE (see Chapter 3.3.3).
- UV-VIS absorption spectra of the supernatants were recorded, to compare the absorbances/concentrations of the expressed cytochrome c_M.

❖ **Chemicals and equipment**

30 % glycerol: see Chapter 3.1.1.10
LB_{KanCm}: See Chapter 3.1.2.9.3
7.27 mM Hemin (M_r = 652.0):
5 mg hemin from Bovine
40 µL 0.5 M NaOH
dissolve hemin in NaOH
add 1 mL steril water and 15 µL 1.0 M HCl
add 0.5 M NaOH to ~ pH 9-10
See Chapter 3.2.1 for further chemicals and equipment

3.2.2.2 Standard protocol

- 30 mL of LB_{KanCm}-medium were inoculated with cells from a cryo culture and incubated overnight at 37°C and 180 rpm.
- 15 mL of this overnight culture were taken as inoculum for 1 L $M9ZB_{KanCm}$-medium, which was divided into two sterile 2 L erlenmeyer flasks.
- The inoculated $M9ZB_{KanCm}$-medium was incubated at 37°C and 180 rpm until OD_{600} reached 1.3.
- Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG.
- The expression culture was incubated for 6 hours at 30°C and 180 rpm and subsequently centrifuged for 10 min at 6000 rpm and 25°C.

- The pellet was resuspended in some supernatant and transferred to four falcon tubes.
- The cell suspension was centrifuged for 15 min at 4000 rpm and 25°C. The supernatant was discarded.
- The cell pellet was stored at –80°C.

❖ **Chemicals and equipment**

See Chapter 3.2.1 with the exception of LB_{KanCm} and $M9ZB_{KanCm}$.

3.3 Purification of recombinant proteins

To avoid blocking the columns with particles and introducing gas bubbles to the systems, all buffers and solutions for chromatographic application were filtrated through 0.45 µm filters using a Nalgene filter-funnel and degassed for 7 min in the ultrasonication bath. Protein solutions were filtrated in the same manner, but degassed under vacuum.

3.3.1 Purification of SUIIc3 cox2

3.3.1.1 Cytoplasmatic release of SUIIc3 cox2

❖ **Principles**

SUIIc3 cox2 was released from the cytosol by lysis of the *E. coli* cells. Protease inhibitors were immediately added to protect the protein from proteolytic digestion. The higher viscosity due to co-release of genomic DNA was reduced by sonication, insoluble proteins and cell components were separated from the soluble protein fractions by centrifugation.

The separation of the recombinant protein from the large amount of soluble *E. coli* proteins in the supernatant was performed by several chromatographic steps.

It is necessary that the reduced state of the two cysteine residues in the active site of the Cu_A domain are preserved for reconstitution of the apoprotein with Cu^{2+}, therefore

DTT, a reducing agent, is added to the lysis buffer and to the buffers used in the first column chromatographic step.

❖ **Protocol**

- The cell pellet of 1 L *E. coli* culture was suspended on ice in 80 mL lysis buffer
- The lysis suspension was sonicated on ice (3 x 40 sec of short pulses) and centrifuged for 20 min at 18 000 rpm and 4°C. The pellet was discarded.
- The supernatant containing the soluble proteins was filtrated and degassed for 15 min using a vacuum flask.

❖ **Chemicals and equipment**

Lysis buffer: See Chapter 3.1.1.9.2
200 mM PMSF and 0.5 M DTT: See Chapter 3.1.1.9.2

Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SS-34 Rotor
Membrane-vacuum pump: vacuubrand CVC 2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (pore size 0.45 µm)

3.3.1.2 Chromatographic purification of SUIIc3 cox2

SUIIc3 cox2 was purified with a two step purification protocol. It comprises an anion exchange chromatography with Q-sepharose and a size exclusion chromatography with Superdex^{TM} 75. Between the two chromatography steps, copper reconstitution was performed (See Chapter 3.3.1.3).
Q-sepharose anion exchange chromatography was performed at 4°C. and Superdex^{TM} 75 size exclusion chromatography at room temperature.
The theoretical isoelectric point of SUIIc3 cox2 is 5.12.

3.3.1.2.1 Anion exchange chromatography with Q-Sepharose Fast Flow

❖ **Principles**

In ion exchange chromatography the separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein is negatively charged and will bind to the positively charged functional groups connected to the matrix of an anion exchanger, when below its pI a protein will bind to a cation exchanger. Q-Sepharose Fast Flow is a strong anion exchanger. Quaternary amine groups $–N^+(CH_3)_3$, which are stable between pH 2 – 12, build the ion exchange groups.
The bound protein can be eluted by changing the conditions like decreasing the pH or increasing the salt concentrations. Those changes can be made stepwise or with a continuous gradient. Usually NaCl gradients are used for elution. During binding target proteins are concentrated and collected in a purified and concentrated form.

❖ **Protocol**

a) Preparation of the column:

- The gel was washed with distilled water in a suction filter and degassed under vacuum.
- Using a syringe, a small volume of degassed water was injected into the column from the bottom side in order to remove trapped air bubbles.
- The column was filled using a glass stick.
- After the gel had sedimented, the column was closed on the top, connected to a peristaltic pump and washed with degassed water until the gel was packed (flow rate: 2 mL/min).
- The prepared column had to be kept in 20 % ethanol.

b) Protein purification

- The column was washed with 2 CV (column volume) of degassed water at a flow rate of 2 mL/min to remove ethanol.
- The column was equilibrated with 2 CV of buffer A at a flow rate of 3 mL/min.

- The sample prepared as described in Chapter 3.3.1 was applied at a flow rate of 3 mL/min.
- The column was washed with 2 CV of buffer A at a flow rate of 3 mL/min.
- The bound protein was eluted by a stepwise increase of NaCl-concentration in buffer A. Elution was performed with 90 mL of step gradient 1, followed by 180 mL of step gradient 2.
- 6 mL fractions were collected.
- After elution the column was washed with 2 CV of buffer B, 2 CV of water at a flow rate of 3 mL/min, and with 2 CV of 20% ethanol at a flow rate of 2 mL/min.
- Fractions were tested for recombinant protein by SDS-PAGE (see Chapter 3.3.3).
- 200 mM PMSF and 0.5 M DTT were added to the pooled fractions to a final concentration of 200 µM PMSF and 0.5 mM DTT.
- The protein is ready for copper reconstitution (See Chapter 3.3.1.3)

❖ **Chemicals and equipment**

20% Ethanol

200 mM PMSF and 0.5 M DTT:

See Chapter 3.1.1.9.2

Buffer A

50 mM phosphate buffer pH 7.0:

6.8 g KH_2PO_4

RO-water added to 1000 mL

pH was tested and adjusted

0.5 mM DTT was added before use

Buffer B:

Buffer A+ 1 M NaCl

0.5 mM DTT was added before use

Step gradient 1:

Buffer A + 100 mM NaCl

0.5 mM DTT was added before use

Step gradient 2:

Buffer A + 200 mM NaCl

0.5 mM DTT was added before use

Column media: Q-Sepharose Fast Flow, Amersham Pharmacia Biotech

stored in 20% ethanol

Column: Pharmacia, ∅ = 2.6 cm; l = 28 cm; V = 150 mL

Peristaltic pump: Pharmacia, Peristaltic Pump P-1; ∅ = 3.1 mm

Membrane-vacuum pump: vacuumbrand CVC2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (Pore size 0.45 µm)
Ultrasonication bath: Bandelin Sonorex Super RK 510 H

3.3.1.2.2 Size exclusion chromatography with Superdex™ 75

❖ **Principles**

The underlying principle of size exclusion chromatography (SEC) is that particles of different sizes, or more precisely different hydrodynamic volum, will elute through a stationary phase at different rates. When gels are used as stationary phases, the technique is more specifically known as gel permeation chromatography (GPC). In a GPC column, gel particles in bead form are packed to an separation bed that is passed by buffer solution, the eluent. Molecules that are to be separated are added in solution to the top of the bed. The sample moves down the bed as eluent is supplied to the top. The small molecules, which diffuse into the gel beads, are delayed in their passage down the column, compared to larger molecules that cannot diffuse into the beads and move continuously down the column in the flowing eluent. The larger molecules thus leave the column first, followed by the smaller ones in the order of their size. In GPC gels the pores have carefully controlled ranges of size, and the matrix is chosen for its inertness (lack of adsorptive properties) as well as its chemical and physical stability.

❖ **Protocol**

For this purification step the ÄktaPurifier FPLC system was used. All steps were carried out at room temperature. The buffers were filtrated and degassed before use and the reconstituted protein solution (See Chapter 3.3.1.3) concentrated to 1 mL with Amicon Centripreps (See Chapter 3.3.1.4). The maximum pressure was set to 1.8 MPa, the UV-detector to 280, 480 and 535 nm and the flow rate to 1 mL/min.

- The (pre-packed) column was washed with 2 CV of degassed water to remove ethanol.

- Subsequently the column was equilibrated with 2 CV of SEC-buffer.
- Before applying the protein solution to the column, it was centrifuged for 5 min at 14000 rpm. 90 µL aliquots of the sample were applied to the column per run, using the 100 µL loop. 10 mL fractions were collected before and 0.3 mL fractions during protein elution.
- Between the runs the column was washed with 10 mL SEC-buffer. After the last run, the column was washed with 2 CV of SEC-buffer and 2 CV of water.
- Subsequently the column was washed with 2 CV of 20% ethanol at an altered flow rate of 0.5 mL/min.
- UV-VIS-absorption-spectra of the fractions were recorded.
- The collected fractions were checked for purity by SDS-PAGE (see Chapter 3.3.3). The protein fractions were pooled and 200 mM PMSF was added to a final concentration of 200 µM.
- The protein pool was concentrated using Amicon Centripreps (see Chapter 3.3.1.4).

❖ **Chemicals and equipment**

SEC-buffer:
9.12 g KH_2PO_3 [67 mM]
11.18 g KCl [150 mM]
6 M NaOH added to pH 7.0
RO-water added to 1000 mL

200 mM PMSF
See Chapter 3.1.1.9.2

20% Ethanol

Column media: Superdex^{TM}75, 24 mL bed volume, exclusion limit: 100 000 Mr (globular proteins), 1.8 MPa maximal back pressure, Pharmacia Biotech
Stored in 20% ethanol

ÄKTApurifier: Pharmacia
Pump P-900
UV-detector UV-900
pH-meter / conductivity cell pH/C-900
Box 900
Fraction collector: Frac-900, Pharmacia

Membrane vacuum pump: vacuumbrand CVC2

Nalgene filter funnel: 315-0047

Millipore filter: HVP04700 (Pore size 0.45 µm)

Ultrasonication bath: Sonorex Super RK 510 H, Bandelin

3.3.1.3 Copper reconstitution

❖ **Principle**

Recombinant SUIIc3 cox2 does not have copper in the active centre as *E. coli* cells show a lack of the required chaperones. In *E. coli* cells Cu^{2+} is bound to different proteins. Otherwise oxygen-radicals would be formed after O_2-activation due to free Cu^{2+} and this would be toxic for the cell.

3.3.1.3.1 Protocol from Synechocystis PCC 6803 for reconstitution of Cu_A domain

- The removed buffer was replaced by 10 mM HEPES buffer, pH 7.5.
- After adding $^1/_5$ of the volume of 50 mM acetate buffer the Cu_A site was reconstituted by adding $Cu(His)_2$ to a final concentration of 1.5 mM.
- Protease inhibitor was added to protect the protein from proteases.
- After 1.5 hours of stirring at 4°C the colour of the solution should change from greenish blue to purple.

See Table 3.21 for a schematic view

Table 3.21: *Schemtaic view of the reconstitution protocol*

3 mL	concentrated Q-sepharose pool of SUIIc3 cox2
97 mL	10 mM HEPES pH 7.5
100 mL	volume of pooled fractions before buffer exchange
20 mL	50 mM NaAc buffer pH 4.6 ($^1/_5$ of the volume)
120 mL	TOTAL VOLUME
1.8 mL	100 mM $Cu(His)_2$
200 µL	200 mM PMSF

❖ **Chemicals and equipment**

10 mM HEPES pH 7.5:
1.19 g HEPES
1 M NaOH added to pH 7.5
RO-water added to 500 mL

50 mM NaAc-buffer pH 4.6:
1.43 mL Glacial acetic acid
1 M NaOH added to pH 4.6
RO-water added to 500 mL

100 mM $Cu(His)_2$:
0.199 g Copper(II)Acetat
0.310 g Histidine
RO-water added to 10 mL

200 mM PMSF: See Chapter 3.1.1.9.2

Magnetic stirrer: IKAMAG RO, Janke & Kunkel, IKA Werk

Stirring bar

3.3.1.3.2 Unfolding by urea and dialysis

An alternative way to reconstitute SUIIc3 cox2 is to unfold the protein with urea and remove the denaturating agent step by step by dialysis.

❖ **Protocol**

- 3 M solid urea, 0.5 M DTT (1:2400) and 200 mM PMSF (1:1000) were added to the unconcentrated Q-Sepharose pool and incubated under stirring at 4°C for 1 hour.
- The dialysis tube was boiled in distilled water for 1 hour.
- The protein solution was filled into the dialysis tube as completely as possible, to still allow sealing but prevent increasing the volume during dialysis as far as possible.
- The dialysis tube was closed with clips.
- The protein solution was dialysed for 1.5 hours under stirring at 4°C in 50 mM phosphate buffer, pH 6.5, and 1 M solid urea with a protein solution to buffer ratio of 1:20.

- The buffer was replaced by a 50 mM phosphate buffer, pH 6.5, without urea and protein solution to buffer ratio of 1:50. The solution was dialysed for 1.5 hours under stirring at 4°C.
- The buffer was replaced by a 50 mM phosphate buffer, pH 6.5, with 1.5 mM $Cu(His)_2$ and protein solution to buffer ratio of 1:20. After dialysis overnight under stirring at 4°C the solution should change from greenish blue to purple.
- The protein was concentrated with Amicon Centripreps (See Chapter 3.3.1.4) and a UV-VIS-spectrum recorded.

❖ **Chemicals and equipment**

50 mM phosphate buffer pH 6.5:
6.8 g KH_2PO_4
RO-water added to 1000 mL
pH was tested and adjusted
0.5 mM DTT was added before use
Urea: Sigma-Aldrich

200 mM PMSF and 0.5 M DTT
See Chapter 3.1.1.9.2

Dialysis Tube: Sigma
Dialysis tubing cellulose membrane
diameter: 25 mm
exclusion limit: 12 000 Da
Clips
Magnetic Stirrer: IKAMAG RET (IKA, Germany)

3.3.1.4 Concentrating protein solution with Amicon Centripreps

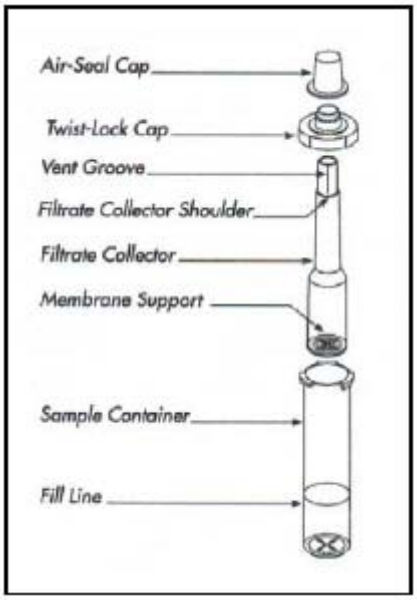
❖ **Principles**

At purification the original volume of a protein solution has to be reduced. Centripreps are based on the principle of ultrafiltration. Their cellulose membrane is permeable for small molecules, whereas bigger components, like proteins, are retained in the sample container. The small molecules are collected in the filtrate collector by centrifugation.

❖ **Protocol**

- 15 mL of protein solution were filled into the sample container.
- The Centriprep was put together and centrifuged for 20 min at 4000 rpm and 4°C.
- The filtrate was discarded.
- Step 1-3 were repeated until the desired volume was reached.

Figure 3.2: *Amicon Centriprep*



❖ **Equipment**

Amicon Centriprep YM-10:
Max. fill volume: 15 mL
End volume: 0.6 - 0.7 mL
Max. RCF: 3000 g
RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA-rotor 1500

3.3.2 Purification of cytochrome c_M

3.3.2.1 Periplasmatic release of cytochrome c_M and dialysis

❖ **Principles**

To release cytochrome c_M from the periplasmatic space of the *E. coli* cells the outer membrane was destroyed using lysozyme. The periplasmic proteins were separated from the *E. coli* spheroblasts by centrifugation and PMSF, a protease inhibitor, was added to inhibit proteolytic degradation. Due to the high lysozyme concentration in the lysis solution, centrifugation had to be performed immediately after resuspension to prevent release of DNA.

Prior to the chromatographic steps, the protein solution had to be dialysed due to the high sucrose concentration in the lysis solution. Dialysis is based on the principle of solutes diffusing along a concentration gradient across a semipermeable membrane. If a solution of different molecules is placed in a dialysis tube (for example a porous cellulose membrane) and the tube is brought into a solution of different concentrations, all molecules small enough to pass the membrane will diffuse in or out of the tube along their concentration gradient. Molecules larger than the pores of the membrane (like proteins) can be retained in the tube while smaller molecules are removed, when a solution of lower concentration is used to dialyse against.

❖ **Protocol**

a) Lysis

- Frozen cell pellets were thawed and 50 mL lysis solution were added per litre initial culture volume (from which the cell pellet was obtained).
- The pellets were resuspended by shaking and stirring with a spatula. Immediately afterwards the suspension was centrifuged 20 minutes at 9000 rpm and 4°C.
- The pellet was discarded. The orange/pinkish coloured supernatant contained the cytochrome c_M.

b) Dialysis

- The dialysis tube was boiled in distilled water for 1 hour.
- The protein solution was filled into the dialysis tube as completely as possible, to still allow sealing but prevent increasing the volume during dialysis as far as possible.
- The dialysis tube was closed with clips.
- The protein solution was dialysed for about 7 hours under stirring at 4°C in 10 mM Tris / HCl pH 8.0 with a protein solution to buffer ratio of 1:40.
- The buffer was removed and the dialysis was resumed overnight with fresh buffer.

❖ **Chemicals and equipment**

Lysis Solution:
50 mM Tris / HCl pH 8
1 mM EDTA
20% Sucrose
500 µg / mL Lysozyme from Chicken Egg White (Sigma)
0.2 mM PMSF

Dialysing Buffer:
10 mM Tris / HCl pH 8
4.84 g Trizma base
3800 mL RO water
6 M HCl added to pH 8
RO water added to 4000 mL

RC-5C Centrifuge: Sorvall Instruments, Du Pont
SLA rotor 1500
Dialysis Tubes: Serva Membra-Cell Dialysis Tubing
MWCO 3500, Diameter 22 mm
Clips
Magnetic Stirrer: IKAMAG RET (IKA, Germany)

3.3.2.2 Chromatographic purification of cytochrome c_M

For cytochrome c_M from *Nostoc* sp. PCC 7120 the same three-step purification protocol as for cytochrome c_M from *Synechocystis* PCC 6803 was chosen. It comprises an anion exchange chromatography with DEAE-sepharose, a cation exchange chromatography with CM-sepharose and a size exclusion chromatography with Superdex^{TM} 75. The theoretical isoelectric point of the protein is 7.04.
All purification steps were performed at room temperature.

3.3.2.2.1 Anion exchange chromatography with DEAE

❖ **Principles**

Diethylaminoethyl (DEAE) sepharose is a weak anion exchanger with $–O\text{-}CH_2CH_3\text{-}N^+(C_2H_5)_2H$ groups bound to a highly cross-linked agarose matrix. It is stable between pH 2 - 9.5. For further principles see Chapter 3.3.1.2.1

❖ **Protocol**

a) Preparation of the column
See Chapter 3.3.1.2.1
b) Protein purification

- The column was washed with 2 CV of degassed water at a flow rate of 2 mL/min to remove ethanol.
- Equilibration of the column was performed with 2 CV of buffer A at a flow rate of 3 mL/min.
- The sample was applied to the column at a flow rate of 3 mL/min.
- The protein was recovered by washing the column with 2 CV of buffer A at 3 mL/min, followed by step gradient 1.
- 6 mL fractions were collected, analysed spectrophotometrically and pooled.

- After elution, the column was washed with 2 CV of buffer B, 2 CV of water, both at a flow rate of 3 mL/min, and 2 CV of 20% ethanol at a flow rate of 2 mL/min.

❖ **Chemicals and equipment**

Buffer A
10 mM Tris / HCl pH 8
1.21 g Trizma base
900 mL RO water
6 M HCl added to pH 8
RO water added to 1000 mL
Buffer B
Buffer A + 1 M NaCl

Step gradient 1:
Buffer A + 0.05 M NaCl
20% Ethanol
Column media: Fractogel EMD 650s DEAE ("tentacle"), Merck

Column: Pharmacia, V = 50 mL

Peristaltic pump: Pharmacia, Peristaltic Pump P-1; ∅ = 3.1 mm

Membrane-vacuum pump: vacuumbrand CVC2
Nalgene filter funnel: 315-0047
Millipore filter: HVP04700 (Pore size 0.45 µm)
Ultrasonication bath: Bandelin Sonorex Super RK 510

3.3.2.2.2 Cation exchange chromatography with CM-Sepharose

❖ **Principles**

In cation exchange chromatography, just in contrary to anion exchange, positively charged proteins bind to the negatively charged functional groups of the chromatography matrix.
Carboxymethyl (CM)-sepharose is a weak cation exchanger with $–CH_3COO^-$ groups bound to a highly cross-linked agarose matrix. The working pH ranges from 3-12. The pH-value of the working buffer is selected in consideration of the pI-value of the target protein. Is the pH-value of the working buffer lower than the pI of the protein, it

turns into its cationic form and binds to the column. The bound proteins can be eluted by increasing the pH or increasing the ionic strength. It is also possible to choose conditions under which not the target protein that is supposed to be purified binds to the column, but certain contaminants. Here this method was used to separate lysozyme (pI = 11) from cytochrome c_M (pI = 6.8).

❖ **Protocol**

a) Preparation of the column

See Chapter 3.3.1.2.1

b) Protein purification

- The column was washed with 2 CV of degassed RO water to remove ethanol (flow rate 2 mL/min).
- The column was equilibrated with 2 CV of buffer A (flow rate 3 mL/min).
- Before application to the column, the protein solution (the concentrated DEAE pool, see 3.5.2) was filtrated and degassed under vacuum for 10 minutes.
- The protein solution was applied at a flow rate of 3 mL/min.
- The column was washed with 2 CV buffer A (flow rate 3 mL/min).
- During sample application and washing the flow-through was collected in 10 mL fractions. It contained cytochrome c_M.
- Lysozyme was eluted from the column with 2 CV buffer B (flow rate 3 mL/min).
- After elution the column was rinsed with 2 CV of buffer B, 2 CV of water and 2 CV of 20% ethanol at 2 mL/min.
- The orange coloured fractions were pooled and concentrated by ultrafiltration to a final volume of 1 mL

❖ **Chemicals and equipment**

Buffer A

50 mM phosphate buffer pH 8:

6.8 g KH_2PO_4

RO-water added to 1000 mL

pH was tested and adjusted

Buffer B

50 mM phosphate buffer pH 8 + 1 M NaCl

20% Ethanol

Column media: : Amersham CM-Sepharose™ Fast Flow

Column: Pharmacia, V = 30 mL

Peristaltic pump: Pharmacia, Peristaltic Pump P-1; ∅ = 3.1 mm

Membrane-vacuum pump: vacuumbrand CVC2

Nalgene filter funnel: 315-0047

Millipore filter: HVP04700 (Pore size 0.45 µm)

Ultrasonication bath: Bandelin Sonorex Super RK 510

3.3.2.2.3 Size exclusion chromatography with Superdex™ 75

❖ **Principles**

See Chapter 3.3.1.2.3

❖ **Protocol**

For this purification step the ÄktaPurifier FPLC system was used. All steps were carried out at room temperature. The buffers were filtrated and degassed before use. The maximum pressure was set to 1.8 MPa, the UV-detector to 280, 410 and 550 nm and the flow rate to 1 mL/min.

The protocol is the same as for SUII c3 cox2 with the exception of the following point:

- The protein pool was concentrated by ultrafiltration (see Chapter 3.3.2.3).

❖ **Chemicals and equipment**

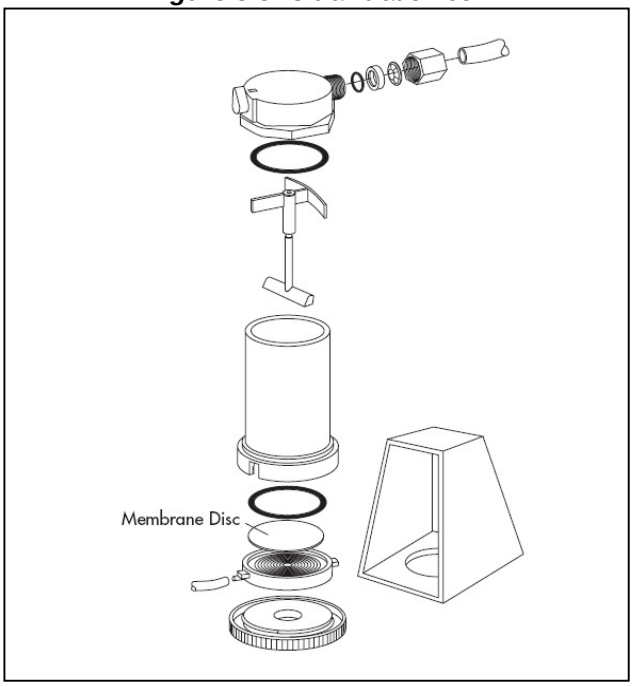
See Chapter 3.3.1.2.3

3.3.2.3 Concentrating protein solution by ultrafiltration

❖ **Principles**

Ultrafiltration is based on the use of regenerated cellulose or polyethersulfone membranes of different pore sizes. Small molecules can pass the membranes, whereas bigger molecules are retained, according to the membranes' molecular weight cut off level. Stirred cells are pressurised devices for ultrafiltration (see Figure 3.6). The pressure forces the fluid to pass the membrane, magnetic stirring prevents concentration polarisation and accumulation of macromolecules on the membrane surface.

Figure 3.3: *Ultrafiltration cell*



❖ Protocol

- The membrane was placed with the glossy side up at the bottom of the ultrafiltration cell.
- All sealings were greased; the cell was assembled and filled with RO water.
- The lid was attached; the device was put into the rack and fixed on a magnetic stirrer.
- Nitrogen was applied to the device via the connection at the lid with a maximum pressure of 4.5 bar. The magnetic stirrer was switched on.
- The membrane was rinsed with water for 10 minutes.
- The remaining water was discarded (gas supply was closed, the pressure discharged), the protein solution was filled in, and the device set up again.
- The pressure was applied until the desired final volume was reached, the concentrated protein solution was removed from the cell using pipettes, and the filtrate was discarded.
- Membranes were stored in 10% ethanol at 4°C.

❖ Equipment

Amicon Stirred Ultrafiltration Cells:

Modell 8010, 10 mL capacity, 1 mL minimal volume, 25 mm membrane diameter

Modell 8200, 200 mL capacity, 5 mL minimal volume, 62 mm membrane diameter

Membranes:

Millipore Ultrafiltration Membranes, Regenerated Cellulose, Filter Code PLBC, NMWC 3000

Magnetic Stirrer: IKAMAG RET (IKA, Germany)

3.3.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

❖ **Principles**

Many biomolecules carrying a charge can move in an electric field. The mobility of the ions depends on charge, size and shape of the molecules. These differences are the basis of electrophoresis.

The electrophoretic separation occurs in inert, homogenous gels (polyacrylamide, agar, starch). The purpose of a gel is to prevent convection of the moving particles and a temperature gradient. Due to the decreased diffusion within the gel, the separated components will move in discrete and separated bands.

Polyacrylamide forms inert, homogenous gels, and exhibits excellent properties for protein separation. The pore size of the gel can be controlled by choosing various concentrations of acrylamide and methylenebisacrylamide (a cross-linking reagent) at the time of polymerisation.

Sodium dodecyl sulfate (SDS) is an anionic detergent, which binds dominantly to proteins causing denaturation. This kind of polyacrylamide gel electrophoresis is one of the most commonly used methods to separate complex protein mixtures and also to determine the relative molecule masses of the proteins.

Under saturated conditions, about 1.4 g SDS are bound per gram protein, all proteins get a certain negative amount of charges per unit of mass. So in most cases the mobility of the protein-SDS-complex, caused by the kind of molecular sieve of the gel, is proportional to the logarithm ($\log_{10}$) of the relative mass (M_r).

To achieve further denaturation of proteins (reduction of disulfide linkages in tertiary and quaternary structure) they may also be boiled in the presence of dithiothreitol (DTT) or 2-mercaptoethanol before application to the gel.

❖ Protocol

a) Preparation of the gel

Mixture for 2 gels: Polymerization ratio: 1:37.5

Table 3.22: *Components of Separating and Stacking Gels.*

	Separating gel	**Stacking gel**
Monomer conc.	15%	4%
RO water	2.29 mL	2.97 mL
1.5 M Tris / HCl	2.50 mL	—
0.5 M Tris / HCl	—	1250 µL
Acrylamide / Bis	5.0 mL	670 µL
10% ($^{w}/_{v}$) SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	10 µL	10 µL

b) Procedure

- The separating gel was cast and covered with 2-butanol to get a plain surface.
- Polymerisation of the gel for 30 min.
- The butanol was discarded, the gel was rinsed with water and the surface was dried using filter paper.
- The stacking gel was added and the comb (10 slots) was set in place.
- Polymerisation for 15 min.
- The comb was removed and the electrophoresis device assembled.
- The gels were put into the electrophoresis chamber that was then filled with 1 x running buffer.
- The samples were diluted 1:2 with 2 x sample buffer and boiled for 4 min.
- 15 µL of each sample and the marker (5 µL) were loaded on the gel using a Hamilton syringe.
- The gel was run at const. 200 V and max. 70 mA

c) Coomassie staining

After the electrophoresis was finished the gel was rinsed with distilled water and incubated in staining solution at room temperature for 30 minutes on a shaker. Then the gel was destained until bands could be visualised with a clear background.

❖ Chemicals and equipment

Acrylamide / Bis:
(30% T; 2.67% C, 1:37.5):
146.0 g Acrylamide
4.0 g Bis
RO-water added to 500 mL
filtrated and stored at 4°C

1.5 M Tris / HCl pH 8.8:
54.45 g Tris base
120 mL RO-water
6 M HCl added to pH 8.8
RO-water added to 300 mL

0.5 M Tris / HCl pH 6.8:
6.0 g Tris base
60 mL water
6 M HCl added to pH 6.8
RO-water added to 100 mL

TEMED

10% (w/v) SDS:
1.0 g SDS in 10 mL RO-water

10% (w/v) APS:
50 mg Ammoniumperoxodisulphate
500 µl RO-water

Marker: Page Ruler^{TM} Unstained Protein Ladder, Fermentas
Broad range: 10-200 kDa.
stored at –20°C

5x Running Buffer, pH 8.3:
15.15 g Tris base
72.0 g Glycine
5.0 g SDS
RO-water added to 1000 mL
stored at 4°C

1x Running buffer, pH 8.3:
160 mL 5x Running buffer, pH 8.3
640 mL RO-water

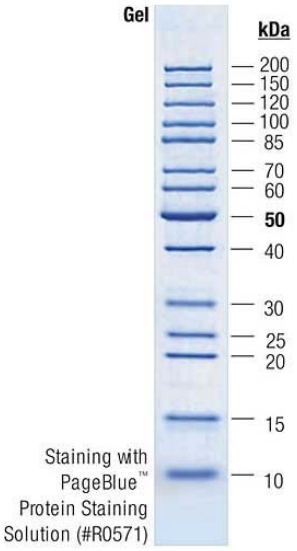
2x Sample buffer stock solution:
2.0 mL Tris / HCl pH 6.8
1.6 mL Glycerol (20%)
3.2 mL 15% SDS (6%)
0.4 mL 0.5% (w/v) Bromphenol Blue

2x Sample buffer:
900 µl 2x Sample buffer stock solution
100 µl β-Mercaptoethanol

Staining solution:
0.1% (w/v) Coomassie Blue R-250
40% (v/v) Methanol
10% (v/v) Glacial actic acid
50% (v/v) RO-water

Destaining solution:
40% (v/v) Methanol
10% (v/v) Glacial actic acid
50% (v/v) RO-water

Figure 3.4: *Page Ruler™ Marker*



BioRad Mini-Protean III Dual Slab Cell

Spacer: 1.00 mm

Voltage source: BioRad Power Supply, Type 500/1000

3.4 Expression, purification and solubilisation of inclusion bodies for reconstitution of SUIIc3 cox2

❖ **Principles**

The overexpression of recombinant proteins sometimes leads to production of inclusion bodies. The protein in the inclusion bodies is fully synthesized, but only partially folded or missfolded. To obtain the correct folded protein, inclusion bodies have to be purified and solubilised with a chaotropic agent, like urea. The denaturating agent is then removed by dialysis.

With inclusion bodies high yield of purified protein can be achieved. Because the denaturating step is also needed to unfold the protein for copper reconstitution of SUIIc3 cox2, it was tried to purify and reconstitute the protein from inclusion bodies.

3.4.1 Expression of inclusion bodies

To promote the expression of inclusion bodies, after induction with IPTG the cells were grown under stress conditions at 42 °C.

❖ **Protocol**

- 40 mL of LB_{AmpCm}-medium were inoculated with cells from a cryoculture and incubated overnight at 37°C and 180 rpm.
- 15 mL of this overnight culture were taken as inoculum for 1000 mL $M9ZB_{AmpCm}$-medium, which was divided into two sterile 2 l erlenmeyer flasks.
- The inoculated $M9ZB_{AmpCm}$-medium was incubated at 37°C and 180 rpm until OD_{600} reached 1.4.
- Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG.
- The expression culture was incubated for 3 hours at 42°C and 160 rpm and subsequently centrifuged for 10 min at 6000 rpm and 25°C (SLA-Rotor).
- The pellet was resuspended in some supernatant and transferred to four falcon tubes.
- The cell suspension was centrifuged for 15 min at 4000 rpm and 25°C. The supernatant was discarded.
- The cell pellet was stored at –80°C.

❖ **Chemicals and equipment**

See Chapter 3.2.1

3.4.2 Purification of inclusion bodies

❖ **Protocol**

a) Lysis

- The cell pellet of 500 mL *E. coli* culture was suspended on ice in 20 mL lysis buffer.

- The lysis suspension was sonicated on ice (3 x 20 sec of short pulses) and centrifuged for 10 min at 8 000 rpm and 4°C. 15 µl supernatant were analysed by SDS-Page, the rest was discarded.
- Step 1 and 2 were repeated for a second time.

b) Purification

- The pellet was suspended on ice in 20 mL 1 M NaCl in RO solution.
- The suspension was sonicated on ice (2 x 20 sec of short pulses) and centrifuged for 10 min at 8 000 rpm and 4°C. 15 µl supernatant were analysed by SDS-Page, the rest was discarded.
- Step 1 and 2 were repeated for a second time.
- The pellet was suspended on ice in 20 mL lysis buffer to eliminate the salt.
- The suspension was sonicated on ice (2 x 20 sec of short pulses) and centrifuged for 10 min at 8 000 rpm and 4°C. 15 µl supernatant were analysed by SDS-Page, the rest was discarded.
- The pellet was suspended on ice in 20 mL RO to eliminate the salt.
- The suspension was centrifuged for 10 min at 8 000 rpm and 4°C. 15 µl supernatant were analysed by SDS-Page, the rest was discarded.
- The last two steps were repeated for a second time

c) Solubilisation

- The pellet was suspended in 20 mL 6 M urea in 50 mM phosphate buffer pH 6.5 solution and incubated for 30 minutes at 4°C under stirring.
- After incubation the solution was centrifuged for 3 min at 14 000 rpm.
- The supernatant was containing the solubilised inclusion bodies.

❖ **Chemicals and equipment**

Lysis solution: See Chapter 3.3.1

1 M NaCl in RO

50 mM phosphate buffer pH 6.5 + 6 M urea

Ultrasonic probe: Sonics & Materials Inc., Vibra-Cell, type CV17

RC-5C Centrifuge: Sorvall Instruments, Du Pont

SS-34 Rotor

3.4.3 Dialysis and copper reconstitution

❖ **Protocol**

- The dialysis tube was boiled in distilled water for 1 hour.
- The protein solution was filled into the dialysis tube as completely as possible, to still allow sealing but prevent increasing the volume during dialysis as far as possible.
- The dialysis tube was closed with clips.
- The protein solution was dialysed overnight under stirring at 4°C in 50 mM phosphate buffer, pH 6.5, and 4 M solid urea with a protein solution to buffer ratio of 1:50.
- The buffer was replaced by 50 mM phosphate buffer, pH 6.5, and 1 M solid urea and protein solution to buffer ratio of 1:50. The solution was dialysed for 3 hours under stirring at 4°C.
- The buffer was replaced by 50 mM phosphate buffer, pH 6.5, without urea and protein solution to buffer ratio of 1:50. The solution was dialysed for 3 hours under stirring at 4°C.
- The buffer was replaced by 50 mM phosphate buffer, pH 6.5, with 1.5 mM $Cu(His)_2$ and protein solution to buffer ratio of 1:50. After dialysis overnight under stirring at 4°C the solution should change from greenish blue to purple.
- Because a lot of protein was denaturated and precipitated, after dialysis the protein solution was centrifuged for 20 min at 4°C and 12 000 rpm.
- The protein solution was concentrated with Amicon Centripreps (See Chapter 3.3.1.4) and a UV-VIS-spectrum recorded.

❖ **Chemicals and equipment**

See Chapter 3.3.1.3.2

3.5 Spectrophotometric investigations

After each purification and reconstitution of the Cu_A-domain UV-VIS absorption spectra of the obtained protein solutions were recorded in order to determine the concentration and reconstitution efficiency.

3.5.1 Spectra of the recombinant proteins

3.5.1.1 Spectrum of the reconstituted, oxidised SUIIc3 cox2

For protein characterization it is necessary to know the positions of the characteristic peaks. Optical spectra (wavelength range: 200 – 900 nm) of the oxidised Cu_A-domains were recorded.
The pure Cu_A domain has a characteristic purple color indicating its oxidised state. SUIIa3 from *Synechocystis* sp. PCC 6803 exhibits two strong absorbance maxima at 482 nm and 535 nm, which arise from the interaction between the two Cu ions. Two additional maxima are present at 359 nm and 785 nm.

The optical spectrum of the reconstituted SUIIc3 cox2 shows characteristic peaks at 480 and 535 nm.

3.5.1.2 Spectrum of oxidised and reduced cytochrome c_M

Optical spectra (wavelength range: 200 – 900 nm) of oxidised and reduced cytochrome c_M were recorded.
Ferrous cytochrome c_M shows three characteristic peaks: α-, β-, and γ-peak (the Soret band). Depending on the oxidative state of the protein, these three peaks show differences in position and intensity (see Table 3.22)

Table 3.23: *Absorption maxima of cytochrome c_M.*

	cytochrome c_M oxidised	cytochrome c_M reduced
α-peak		550 nm
β-peak		521 nm
γ-peak	406 nm	416 nm

3.5.2 Determination of the molar extinction coefficient $\varepsilon_{280\ nm}$

3.5.2.1 Determination of the molar extinction coefficient $\varepsilon_{280\ nm}$ for SUIIc3 cox2

The extinction coefficient ε at 280 nm was calculated on the basis of the amino acid sequence with ExPASy Prot Param software tool.

3.5.2.2 Determination of molar extinction coefficients for cytochrome c_M

- Protein solutions with 6, 12, 18, 24 and 30 µM cytochrome c_M in its oxidized state were prepared to record absorbance spectra.
- Three spectra for each concentration were recorded.
- The mean of the absorbances was plotted against the concentration.
- The extinction coefficient was calculated from the slope of the linearly fitted calibration curve using the Lambert Beer's Law.

❖ **Chemicals and equipment**

Cytochrome c_M in SEC-buffer, pH 7
Spectrophotometer: Diode Array Specord UV-VIS S 10, Zeiss Instruments
Software: Aspect plus version 1.5
Cuvettes: Quartz-cuvettes (1 cm light-path)
Centrifuge: Sigma 1-15 (Sigma, Germany)

3.5.3 Determination of protein concentration

❖ **Principles**

From the measured absorptions at specific wavelengths the protein concentration can be calculated according to Lambert-Beer's Law using the molar extinction coefficient ε.

Lambert-Beer's Law: $A = c \times \varepsilon \times d$

A … absorption
c … concentration [mol / l]
ε … molar extinction coefficient [$M^{-1}cm^{-1}$]
d … diameter of the cuvette: 1 cm

3.5.3.1 Determination of SUIIc3 cox2 protein concentration

❖ **Protocol**

- The sample was centrifuged at 14000 rpm for 4 min at RT, and if necessary it was diluted before the measurement.
- The SUIIc3 cox2 holoprotein concentration was determined with the molar extinction coefficients of SUIIa3 of *Synechocystis* PCC 6803 (see Table 3.24).

Table 3.24: *Extinction coefficients of Cu_A-domain*

ε (wavelength)	Value
$\varepsilon_{480\ nm}$	2.8 $mM^{-1}\ cm^{-1}$
$\varepsilon_{535\ nm}$	3.1 $mM^{-1}\ cm^{-1}$

- The total protein concentration of SUIIc3 cox2 was determined with the molar extinction coefficient at 280 nm (for determination of $\varepsilon_{280\ nm}$ see Chapter 3.5.1.1)

$\varepsilon_{280\ nm}$ of SUIIc3 cox2 = 21.0 $mM^{-1}\ cm^{-1}$

❖ **Equipment**

Spectrophotometer: Diode Array Specord UV-VIS S 10, Zeiss Instruments
Software: Aspect plus version 1.5
Cuvettes: Quartz-cuvettes (1 cm light-path)
Centrifuge: Sigma 1-15 (Sigma, Germany)

3.5.3.2 Determination of cytochrome c_M protein concentration

❖ **Protocol**

- The sample was centrifuged at 14000 rpm for 4 min at RT, and if necessary it was diluted before the measurement.
- The protein concentration of cytochrome c_M was determined with the molar extinction coefficient at 280 nm (for determination of $\varepsilon_{280\ nm}$ see Chapter 3.5.1.2)

$$\varepsilon_{280\ nm} \text{ of cytochrome } c_M = 17.6\ mM^{-1}\ cm^{-1}$$

❖ **Equipment**

See Chapter 3.5.2.1

3.5.4 Reduction of cytochrome c_M

Unlike cytochrome c_M from *Synechocystis* PCC 6803, cytochrome c_M from *Nostoc* sp. PCC 7120 is almost completely oxidised after cell lysis and oxidises completely during the purification procedure.
The capability of reduction was tested with a strong reduction agent (sodium-dithionite) and a weaker one (sodium-ascorbate).

3.5.4.1 Reduction with sodium-dithionite

❖ **Protocol**

- Solid sodium-dithionit was added to the protein solution. The cuvette was covered with Parafilm, its content was mixed by inverting.
- A UV-VIS absorption spectrum was recorded in the range of 200 – 800 nm.

3.5.4.2 Reduction with sodium-ascorbate

❖ **Protocol**

- Sodium-ascorbat was added to the protein solution beginning with a 10 fold surplus. The content was mixed by inverting the cuvette.
- The quantity of sodium-ascorbate was increased step by step trying to reduce the protein solution.
- UV-VIS absorption spectra were recorded.

❖ **Chemicals and equipment**

Sodium dithionite: $Na_2S_2O_4$ (Sigma Aldrich)
0.5, 5 and 10 mM Sodium Ascorbate
Spectrophotometer: Diode Array Specord UV-VIS S 10, Zeiss Instruments
Software: Aspect plus version 1.5
Cuvettes: Quartz-cuvettes (1 cm light-path)
Centrifuge: Sigma 1-15 (Sigma, Germany)

4. Results

4.1 Cloning of recombinant proteins

The soluble part of cytochrome *c* oxidase 2 subunit II (SUIIc3 cox2) and cytochrome c_M of *Nostoc* sp. PCC 7120 were cloned in different *E. coli* strains to allow recombinant expression of the proteins.

4.1.1 Cloning of SUIIc3 cox2

E. coli BL21(DE3)pLysS were successfully transformed with the pET-3a plasmid carrying the cytochrome *c* oxidase 2 subunit II insert (amino acid residues 113-327).

4.1.1.1 Amplification by PCR

SUIIc3 cox2 was amplified by PCR. The obtained PCR-fragment was purified with Gene JET™ Plasmid Mini Prep-Kit. To verify the length of the insert agarose gel electrophoresis was performed. Figures 4.1 shows the gel with the DNA visualised under UV-light.
The estimated length of the fragment from the agarose gel conforms to the calculated length (655 bp) of SUIIc3 cox2.

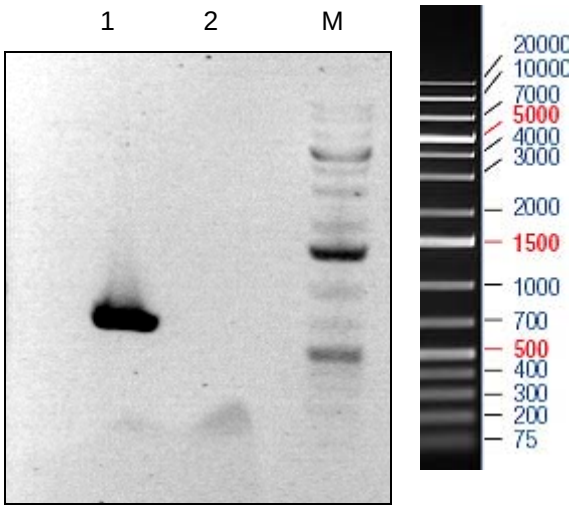


Figure 4.1: *Agarose gel electrophoresis of SUIIc3 cox2 after amplification by PCR; lane 1: SUIIc3 cox2, lane 2: negative control, lane M: 1 kb ladder*

4.1.1.2 Digestion of insert and vector DNA and ligation

The purified insert coding for SUIIc3 cox2 and the pET-3a vector were digested with the restriction enzymes *Bam*HI and *Nde*I. Alkaline phosphatase was added to the digestion approach of vector DNA to prevent ligation of vector DNA without an insert. Agarose gel electrophoresis (Figure 4.2) was performed in order to determine the concentration before ligation.

Vector and insert DNA were ligated by T4-DNA-ligase.

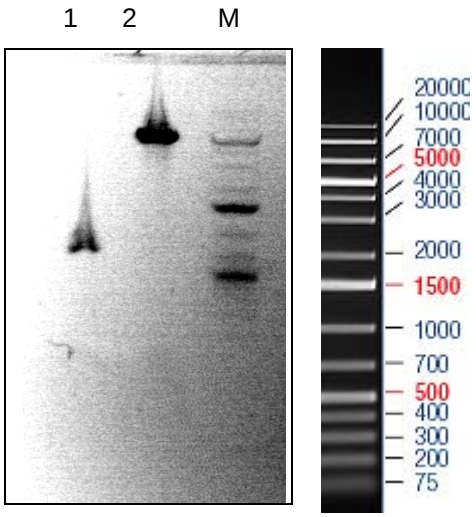


Figure 4.2: *Agarose gel electrophoresis of digested insert and pET-3a vector DNA; lane 1: insert DNA, lane 2: pET-3a vector, lane M: 1 kb ladder*

4.1.1.3 Screening for positive clones

After transformation by electroporation, screening for positive clones was performed by different techniques. First, colonies on the masterplate were analysed by PCR screening. An expression screening was then performed to confirm that the clone is also expressing the desired protein.

Agarose gel electrophoresis (Figure 4.3) shows a PCR product of the right length indicating a positive clone.

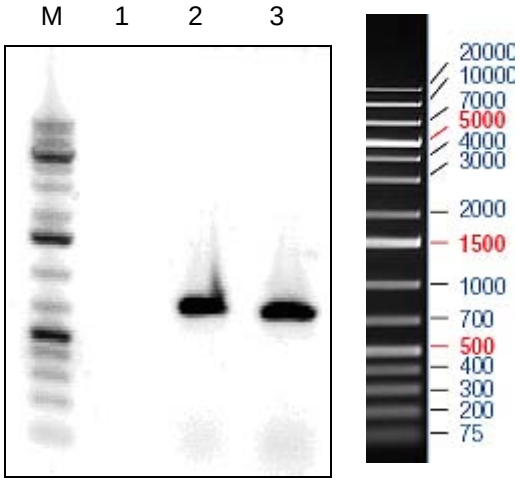


Figure 4.3: *Agarose gel electrophoresis of PCR screening; lane M: 1 kb ladder, lane -: negative control; lane 2: positive control; lane 3: positive clone.*

A small-scale expression of the positive clone was performed to investigate its expression abilities. The molecular weight of the expressed proteins was checked by SDS-PAGE and compared to the theoretical molecular weight of the SUIIc3 cox2 fragment. The SDS-PAGE (Figure 4.4) shows that the c3-clone of SUII cox2 is expressing a good amount of soluble protein. A little amount of protein remains in the pellet.

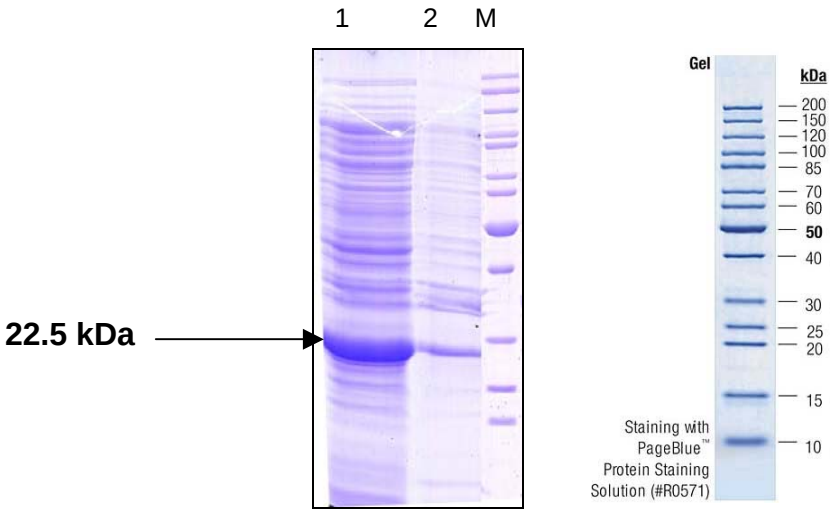


Figure 4.4: *SDS-PAGE of the expression screening; lane 1: supernatant with soluble protein, lane 2: pellet; lane M: marker*

4.1.1.4 Sequencing of plasmid DNA

The sequence of clone 3 of SUII cox2 and was determined to assure that the plasmid DNA does not contain mutations.

The DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Plasmid DNA was purified with Gene Jet™ Plasmid Mini-Prep Kit and T7 promoter and terminator primer were provided. Agarose gel electrophoresis was performed to estimate plasmid DNA concentration (Figure 4.7). The software tool DNAStar SeqMan 4.0 was used to compare the obtained sequence with the theoretical one.

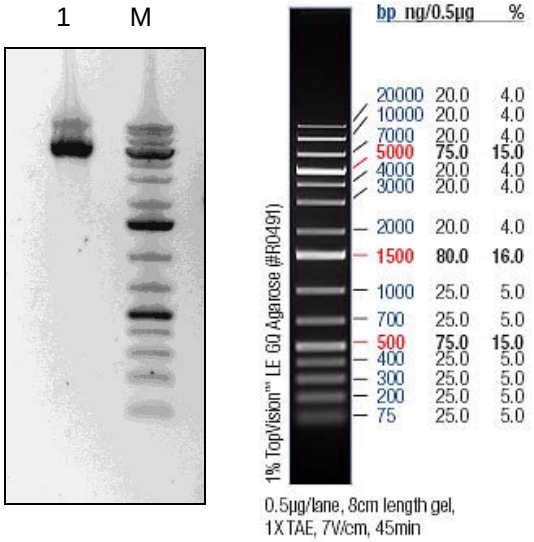


Figure 4.5: *Agarose gel electrophoresis of the plasmid DNA of SUIIc3 cox2; lane 1: 2 µL SUIIc3 cox2; lane M: 1 kb ladder*

No mutations were found that led to alterations of the proteins' amino acid sequence.

4.1.2 Cloning of cytochrome c_M

Cytochrome c_M was also successfully cloned in *E. coli* BL21(DE3)Star carrying the pEC86 plasmid, using the pET-27b+ plasmid.

4.1.2.1 Amplification by PCR

Cytochrome c_M was amplified by PCR. The obtained PCR-fragment was purified with Gene JET™ Plasmid Mini Prep-Kit. To verify the length of the insert agarose gel

electrophoresis was performed. Figures 4.6 shows the gel with the DNA visualised under UV-light.

The estimated length of the fragment from the agarose gel conforms to the calculated length (231 bp) of cytochrome c_M.

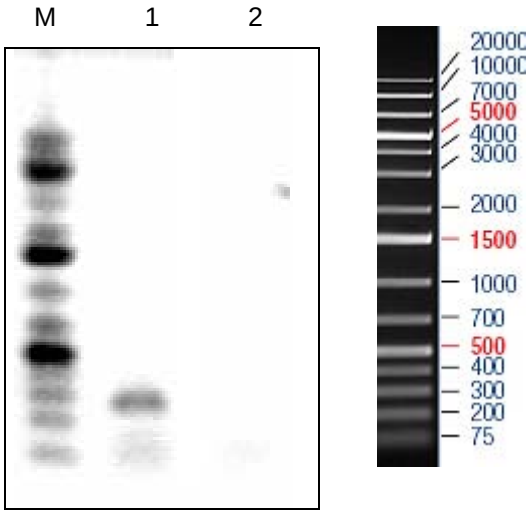


Figure 4.6: *Agarose gel electrophoresis of cytochrome c_M after amßplification by PCR; lane M: 1 kb ladder, lane 1:, lane 2: cytochrome c_M negative control*

4.1.2.2 Digestion of insert and vector DNA and ligation

The purified insert coding for cytochrome c_M and the pET-27b+ vector were digested with the restriction enzymes *Nco*I and *Bam*HI. Alkaline phosphatase was added to the digestion approach of vector DNA to prevent ligation of vector DNA without an insert. Agarose gel electrophoresis (Figure 4.7) was performed in order to determine the concentration before ligation.

Vector and insert DNA were ligated by T4-DNA-ligase.

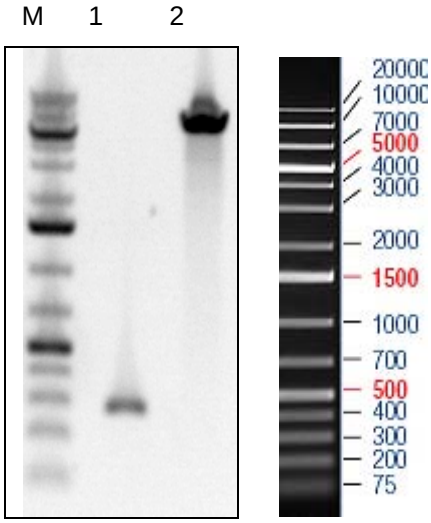


Figure 4.7: *Agarose gel electrophoresis of digested insert and pET-27b^{+} vector DNA; lane 1: insert DNA, lane 2: pET-27b+ vector, lane M: 1 kb ladder*

4.1.2.3 Screening for positive clones

Colonies on the masterplate were analysed by PCR screening. An expression screening was then performed to confirm that the clone is also expressing the desired protein.

Agarose gel electrophoresis (Figure 4.8) shows three positive clones found by PCR screening at the right length.

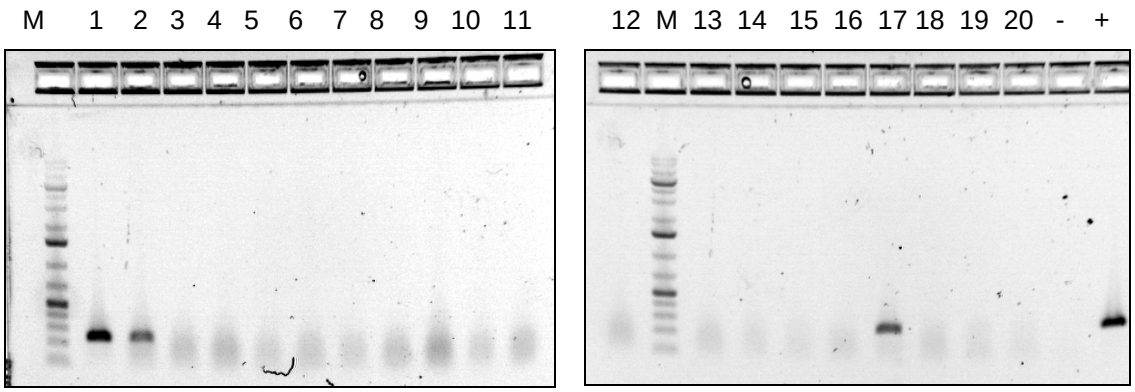


Figure 4.8: *Agarose gel electrophoresis of PCR screening; lane M: 1 kb ladder, lane 1, 2 and 17: positive clones; lane +: positive control; lane -: negative control.*

A small-scale expression of the positive clones was performed to investigate its expression abilities. The molecular weight of the expressed proteins was checked by

SDS-PAGE and compared to the theoretical molecular weight of cytochrome c_M. UV-VIS spectra were also recorded.

SDS-PAGE (Figure 4.9) shows that significant amount of protein remains in the pellet as non-soluble protein. Only a little amount of protein is expressed as soluble protein. The clones do not show relevant difference in the expressed amount.

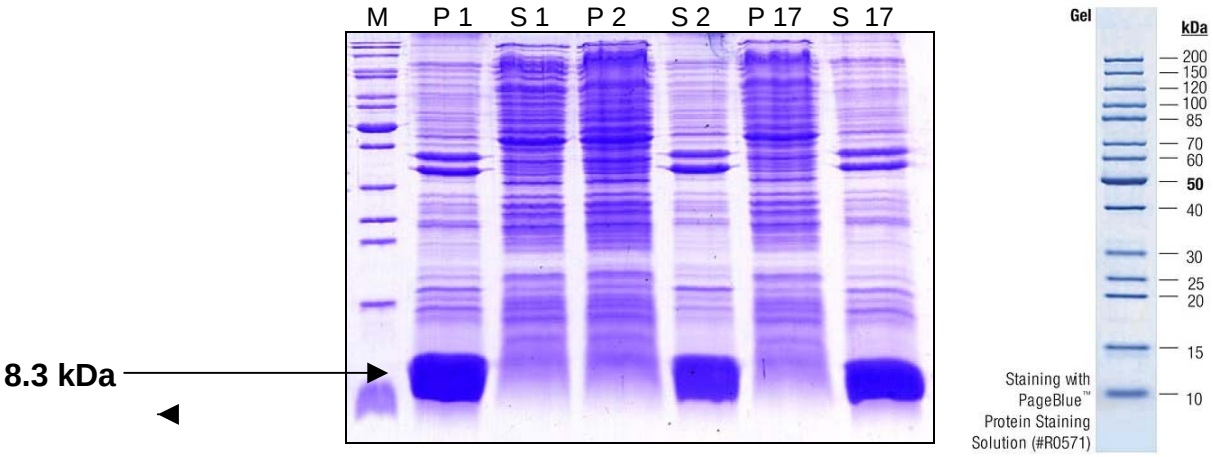


Figure 4.9: *SDS-PAGE of the expression screening; lane M: marker, lane P1, P2, P17: pellet of clone 1, 2, and 17, lane S1, S2 and S17: supernatant of clone 1, 2 and 17.*

The overlay of the spectra of the three clones (Figure 4.10) shows that clone 1 produces the largest amount of soluble protein.

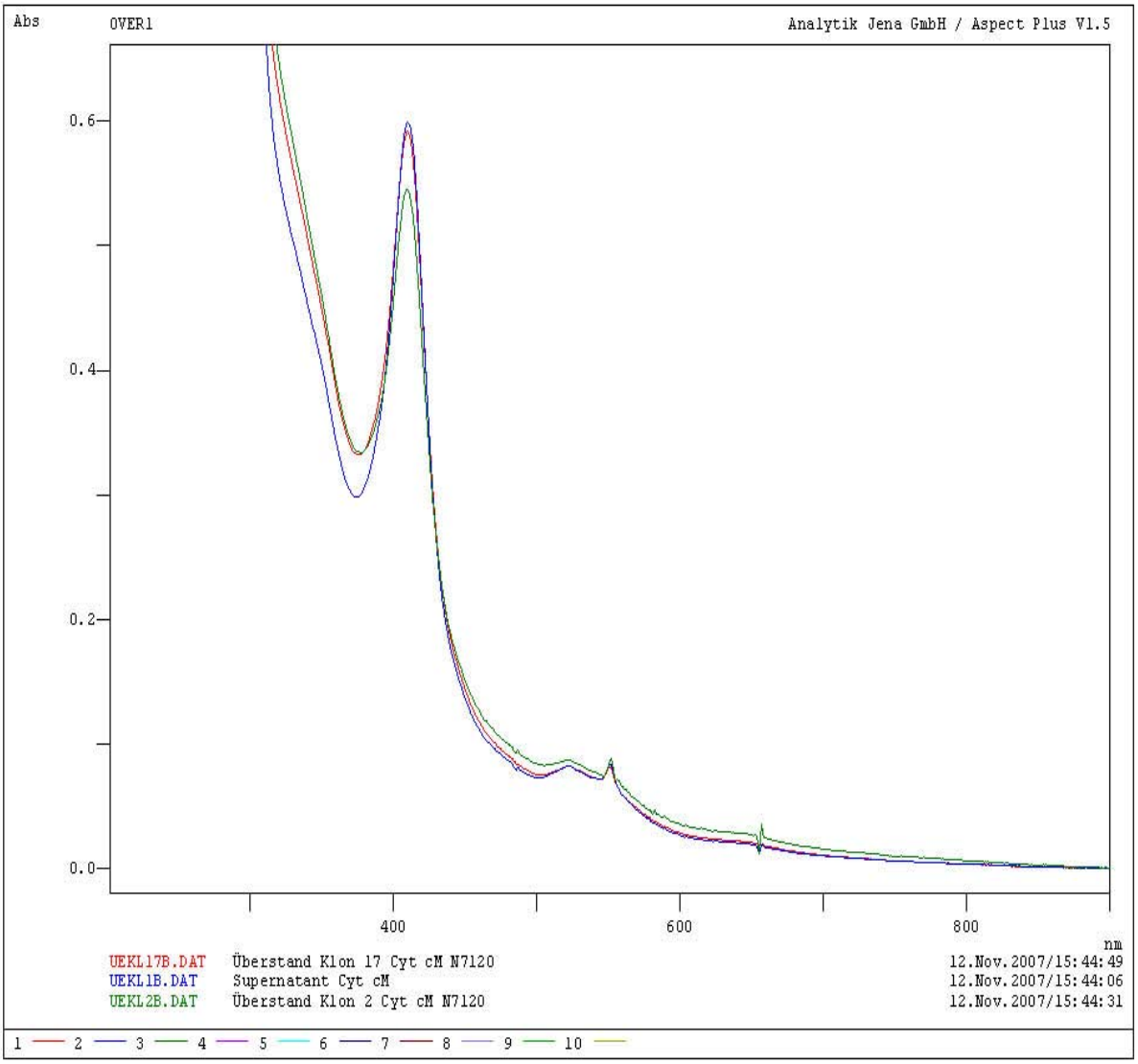


***Figure 4.10: Overlay of the** UV-VIS spectra of the three clones expressing cytochrome c_M; in red clone 1, in green clone 2 and in blue clone 17. Clone 1 (in red) expresses the largest amount of soluble protein.*

4.1.2.4 Sequencing of plasmid DNA

The DNA sequencing was conducted by I.B.L., Dennisgasse 23, 1200 Vienna. Plasmid DNA was purified with Gene Jet™ Plasmid Mini-Prep Kit and T7 promoter and terminator primer were provided. Agarose gel electrophoresis was performed to estimate plasmid DNA concentration (Figure 4.11). The software tool DNAStar SeqMan 4.0 was used to compare the obtained sequence with the theoretical one.

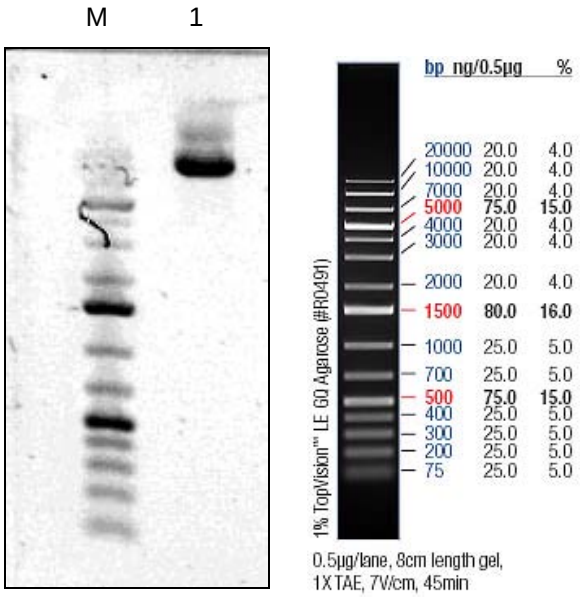


Figure 4.11: *Agarose gel electrophoresis of the plasmid DNA of cytochrome c_M; lane M: 1 kb ladder; lane 1: 2 µL cytochrome c_M*

No mutations were found that led to alterations of the proteins' amino acid sequence.

4.2 Expression of recombinant proteins

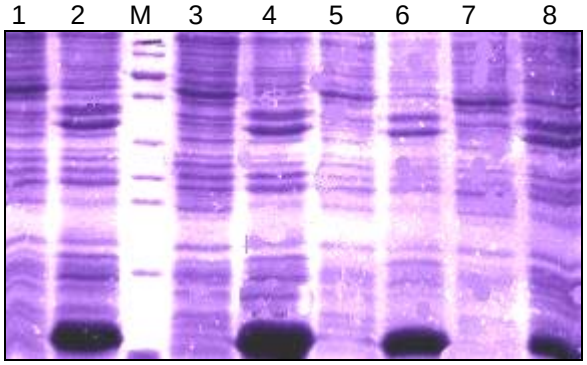
4.2.1 Expression of SUIIc3 cox2

The SUIIc3 cox2 was expressed in the *E. coli* strain BL21(DE3)pLysS using a protocol similar to that of SUIIj3 cox2: 2 L of $M9ZB_{AmpCm}$ were inoculated with 24 mL overnight culture prepared from a cryo culture. The cells were incubated at 37°C and 180 rpm until OD_{600} 1.4 was reached. Subsequently the expression was induced by the addition of IPTG to a final concentration of 1 mM IPTG. The expression culture was incubated for 20 h at 16°C and 180 rpm. A longer expression time at low temperature promotes the correct folding of the protein.

Using this protocol, copper reconstitution by dialysis and the purification protocol described in Chapter 3.3.1 an average of 10 mg total protein could be obtained per litre culture volume, but only 20% of this amount could be obtained as holoprotein (see Chapter 4.3.1.2).

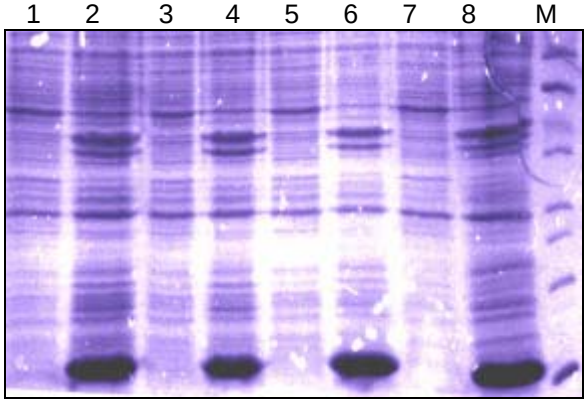
4.2.2 Expression of cytochrome c_M

Expression screening (see Chapter 4.1.2.3) showed that only a little amount of protein is produced. To optimise the culture conditions of the expression of cytochrome c_M preliminary test were performed with varying media, duration of expression, incubation temperatures, IPTG, glycerol and hemin additions.
Supernatant and pellet were analysed by SDS-PAGE and UV-VIS spectra of the supernatant were also recorded.
Figure 4.12a and 4.12b shows the SDS-PAGE of a couple of tests performed, No relevant differences between the clones are detectable with electrophoresis, but expression with M9ZB$_{KanCm}$, 1 mM IPTG at 16 and 30°C seems to work best.



M9ZB$_{kanCm}$ Medium + 1 mM IPTG	
1	Supernatant 16 °C
2	Pellet 16 °C
M	Marker
3	Supernatant 20 °C
4	Pellet 20 °C
5	Supernatant 30 °C
6	Pellet 30 °C
7	Supernatant 37 °C
8	Pellet 37 °C

Figure 4.12a: *SDS-SPAGE of some preliminary tests with M9ZB$_{kanCm}$ at different expression temperatures and 1 mM IPTG*



LB$_{kanCm}$ Medium + 1 mM IPTG + 0.2% Glycin	
1	Supernatant 16 °C
2	Pellet 16 °C
3	Supernatant 20 °C
4	Pellet 20 °C
5	Supernatant 30 °C
6	Pellet 30 °C
7	Supernatant 37 °C
8	Pellet 37 °C
M	Marker

Figure 4.12b: *SDS-PAGE of some preliminary tests with LB$_{kanCm}$ at different expression temperatures, 1 mM IPTG and 0.2% Glycerol.*

Figure 4.13 shows the UV-VIS spectra of the corresponding preparations. Expression at 30 °C with M9ZB$_{kanCm}$ and 1 mM IPTG produced the largest amount of

intact heme protein. Using this protocol, after purification an average of 1 mg total protein could be obtained per litre culture volume.

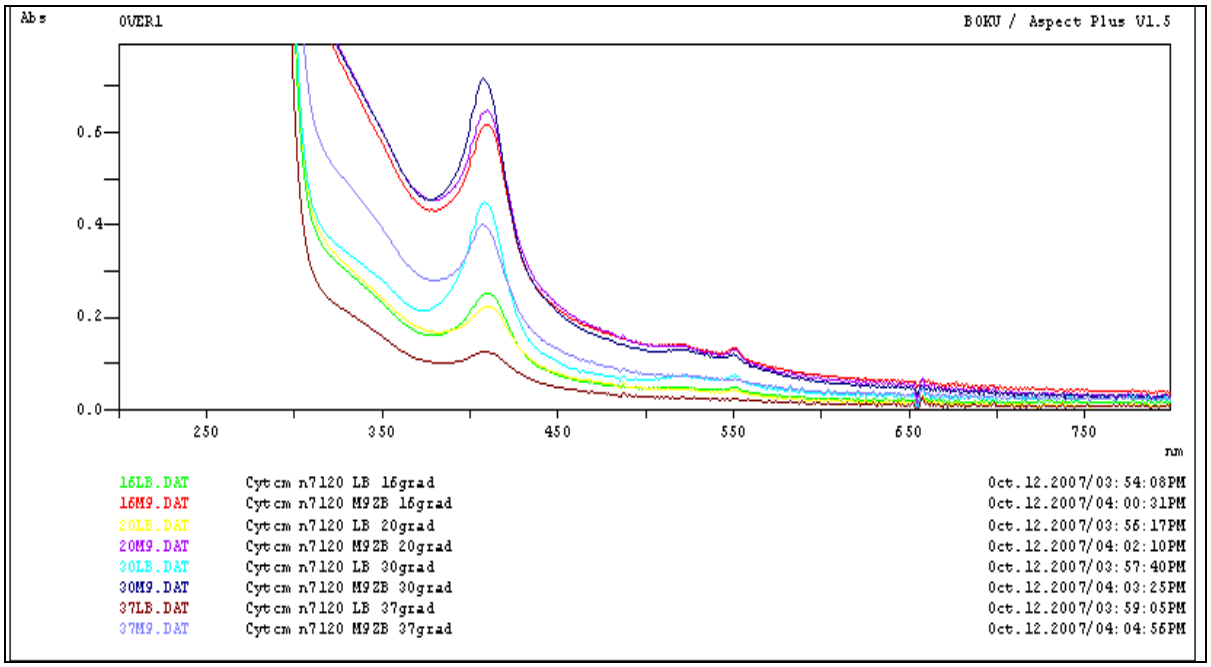


Figure 4.13: *UV-VIS spectra of some preliminary test with M9ZB$_{kanCm}$ at different expression temperatures and 1 mM IPTG; LB$_{kanCm}$ at different expression temperatures, 1 mM IPTG and 0.2% Glycerol.*

4.3 Purification of recombinant proteins

4.3.1 Purification of SUIIc3 cox2

4.3.1.1 Chromatograpic purification

The overexpressed protein was released from the cytosol by lysis of the *E. coli* cells. Protease inhibitor was immediately added to protect the proteins from proteolytic digestion. It is necessary that the reduced state of the two cysteine residues in the active site of the Cu_A domain is preserved for reconstitution of the apoprotein with Cu^{2+}, therefore DTT, a reducing agent, is added to the lysis buffer and to the buffers of the first column chromatographic step before copper reconstitution.

SUIIc3 cox2 was purified by two chromatographic steps: anion exchange chromatography with Q-Sepharose Fast Flow and by size exclusion chromatography

with Superdex^{TM} 75. Between the two chromatographic steps copper reconstitution was performed.

Figure 4.14 shows selected fractions after the Q-Sepharose Fast Flow chromatography. SUIIc3 cox2 elute with step gradient 1 (buffer A + 100 mM NaCl), at step gradient 2 (buffer A + 200 mM NaCl) a small amount of SUIIc3 cox2 and high amount of other protein is present. Only fraction with a high amount of SUIIc3 cox2 and a small amount of other protein are pooled. In this case fractions 1-13 were pooled, PMSF and DTT were added to the protein pool to a final concentration of 200 µM PMSF and 0.5 mM DTT. The protein was afterwords reconstituted (see Chapter 4.3.1.2).

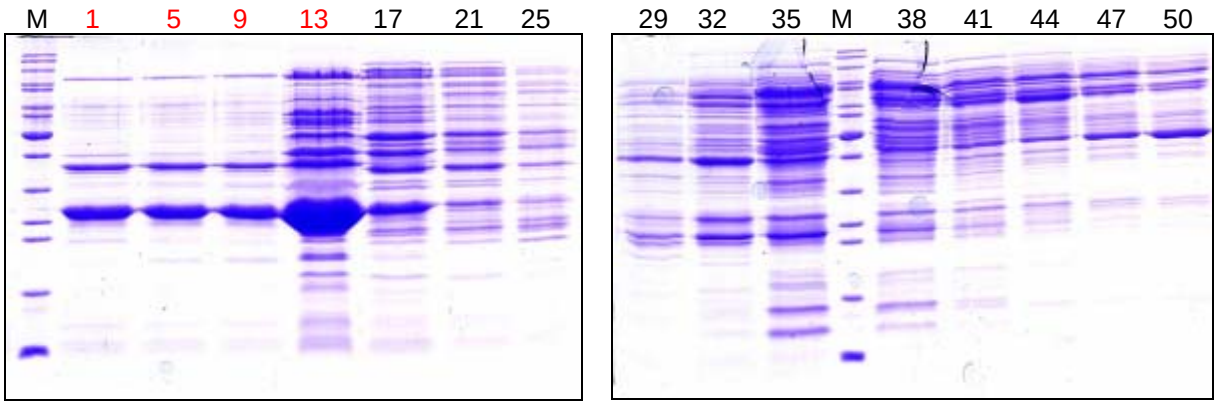


Figure 4.14: *SDS-PAGE of SUIIc3 cox2 after Q-Sepharose Fast Flow*
Numbers 1-50 indicate the fractions collected during elution; fractions numbers pooled are coloured red; lane M: marker

Figure 4.15 shows a typical chromatogramm of the purification step with Superdex^{TM} 75.

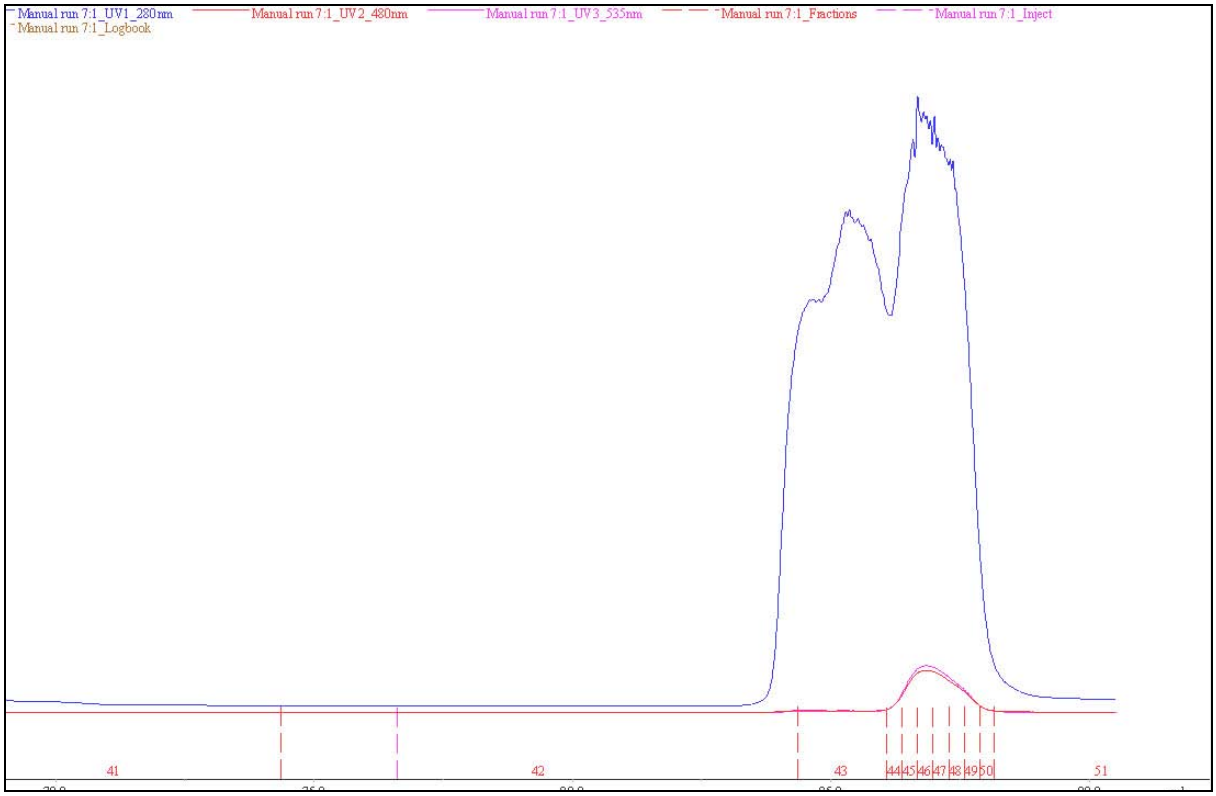


Figure 4.15: *Chromatogramm of SUIIc3 cox2 of the Superdex™ 75 chromatographical step. In blue the spectrum at 280 nm, in red the spectrum at 480 nm and in blue at 535 nm.*

The protein fractions from the beginning to the end of the elution peak (shown in Figure 4.15) were analysed by SDS-PAGE (4.16). The later fractions contain more pure protein than the earlier one. Only fractions 46-49 were pooled.

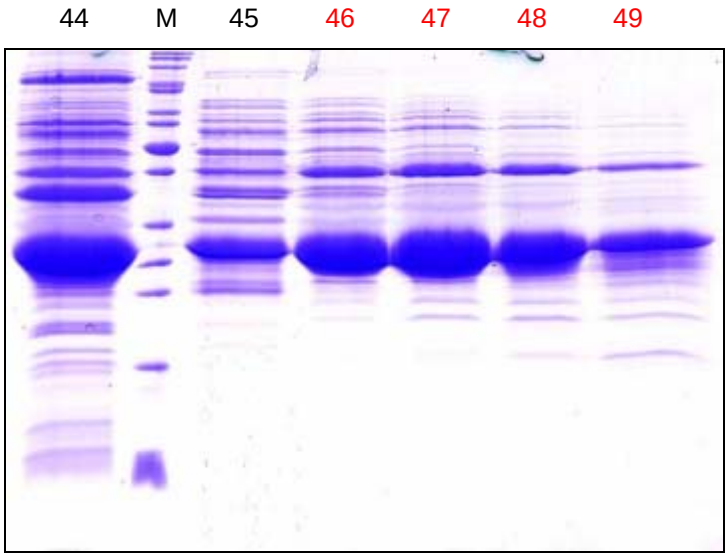


Figure 4.16: *SDS-PAGE of the protein fractions from the beginning to the end of the peak. Lane 44-49: fractions from the beginning to the end of the peak; lane M: marker. The fractions numbers in red were pooled.*

The SUIIc3 cox2 solution obtained with this purification protocol was not yet pure and contained contaminant proteins.

4.3.1.2 Copper reconstitution

Recombinant SUIIc3 cox2 does not have copper in the active centre as *E. coli* cells show a lack of the required chaperones. The reconstituted, oxidized Cu_A domain is characterised by its purple colour and exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands.
Two approaches were tried to reconstitute the protein: the protocol from the Cu_A-domain of *Synechocystis* PCC 6803 and dialysis after unfolding with urea.

4.3.1.2.1 Protocol from Synechocystis PCC 6803 for reconstitution of Cu_A domain

The protein pool after Q-sepharose was first concentrated with Amicon Centripreps and the removed buffer replaced by 10 mM HEPES buffer. After adding $^1/_5$ of the volume of 50 mM acetate buffer the Cu_A site was reconstituted by adding $Cu(His)_2$ to a final concentration of 1.5 mM. Protease inhibitor was added to protect the protein from proteases. After 1.5 hours of stirring at 4°C the colour of the solution should change from greenish blue to purple.
No colour change was observed, therefore it was not possible to reconstitute the protein by using this protocol.

4.3.1.2.2 Unfolding by urea and dialysis

An alternative way to reconstitute SUIIc3 cox2 is to unfold the protein with urea and refold the protein by removing the denaturating agent step by step by dialysis.
This was achieved by incubating the unconcentrated Q-Sepharose protein pool with 3 M urea at 4 °C for 1 hour. The protein solution was then filled into a dialysis tube and dialysed for 1.5 hours at 4°C in 50 mM phosphate buffer, pH 6.5, and 1 M urea with a protein solution to buffer ratio of 1:20. The buffer was then replaced with a 50 mM phosphate buffer, pH 6.5, without urea and protein solution to buffer ratio of

1:50. The solution was dialysed for 1.5 hours under stirring at 4°C. The buffer was once again substituted by a 50 mM phosphate buffer, pH 6.5, with 1.5 mM $Cu(His)_2$ and protein solution to buffer ratio of 1:20. After overnight dialysis at 4°C the solution showed a slightly purple color.

The reconstituted SUIIc3 cox2 solution was concentrated with Amicon Centripreps. During concentration the $Cu(His)_2$ solution was washed out and the solution became more and more purple. The recorded UV-VIS spectrum (Figure 4.17) showed the typical absorbance maxima at about 480 nm and 535 nm but only 20% of total SUIIc3 cox2 was reconstituted.

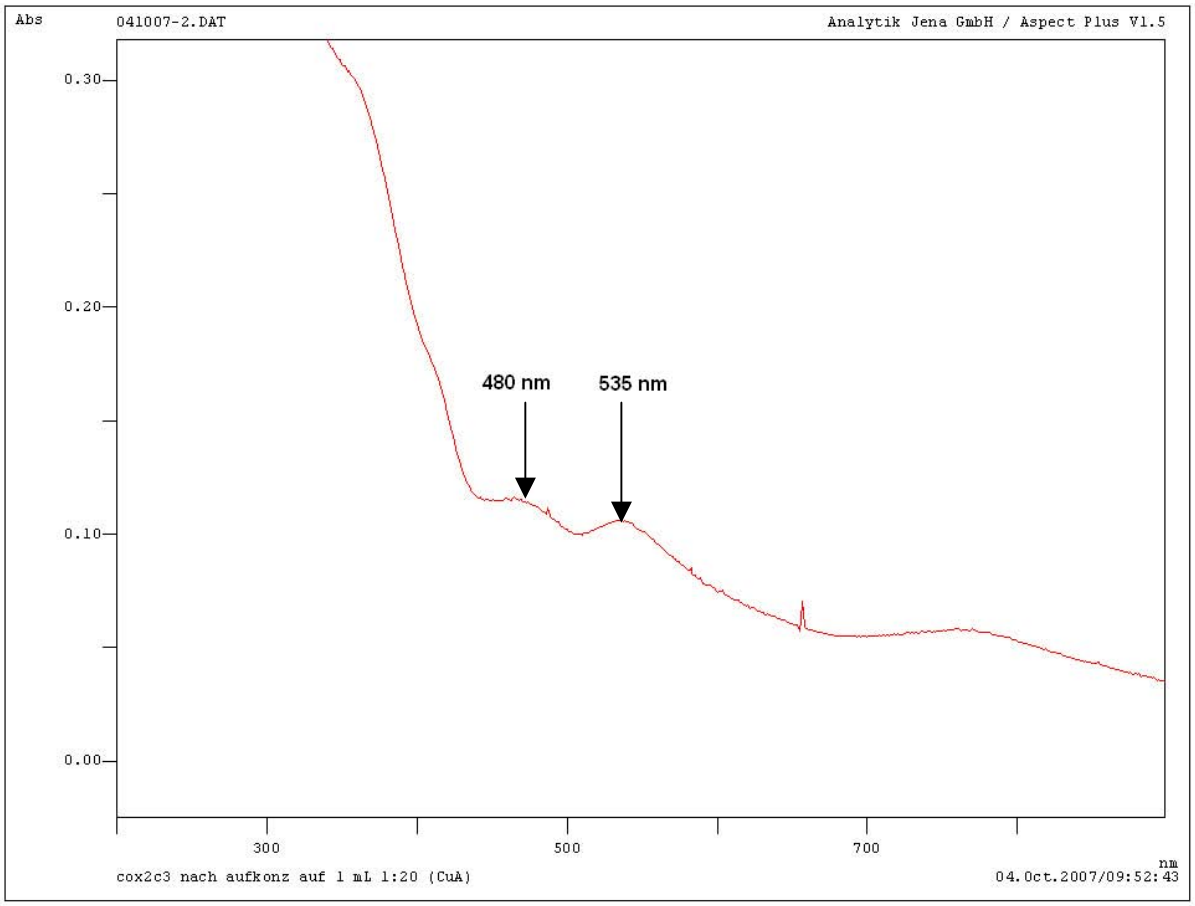


Figure 4.17: *UV-VIS spectrum of the reconstituted Cu_A-domain.*

4.3.2 Purification of cytochrome c_M

To release cytochrome c_M from the periplasmatic space of the *E. coli* cells the outer membrane was destroyed using lysozyme. The periplasmic proteins were separated from the *E. coli* spheroblasts by centrifugation and PMSF, a protease inhibitor, was added to inhibit proteolytic degradation. The protein solution was then dialysed and purificated with a three-step procedure: an anion exchange chromatography with DEAE-sepharose, a cation exchange chromatography with CM-sepharose and a size exclusion chromatography with Superdex^{TM} 75.

Figure 4.18 shows the SDS-PAGE of the three purification steps: CM-sepharose removes lysozyme, but only after the size exclusion chromatography with Superdex^{TM} 75 the contaminant proteins are removed.

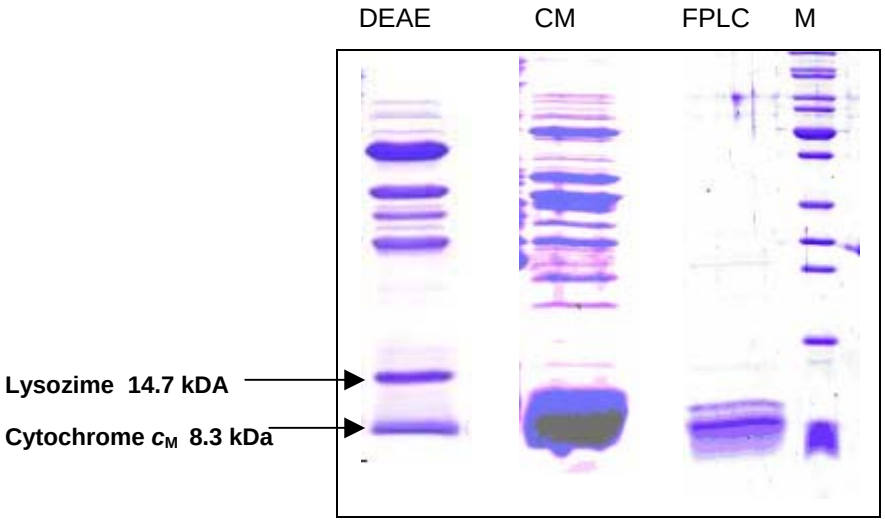


Figure 4.18: *SDS-PAGE of the three purification steps. DEAE: DEAE-sepharose; CM: CM-sepharose; FPLC: Superdex^{TM} 75; M: Marker*

Figure 4.19 shows a typical chromatogramm of the purification step with Superdex^{TM} 75 chromatography.

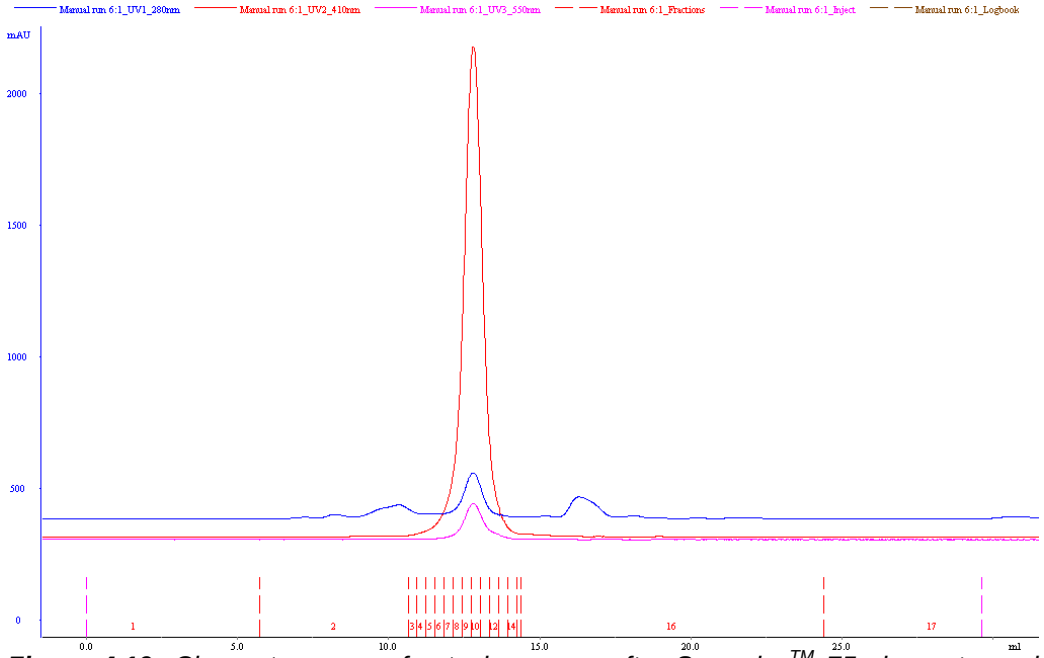


Figure 4.19: *Chromatogramm of cytochrome c_M after Superdex^{TM} 75 chromatography. In blue the spectrum at 280 nm, in red the spectrum at 416 nm and in blue at 550 nm.*

The cytochrome c_M solution obtained via this three-step purification was of high purity and contained almost no other proteins.

4.4 Expression, purification and solubilisation of inclusion bodies for copper reconstitution of SUIIc3 cox2

SDS-PAGE of the expression screening (see Chapter 4.1.1.3 Figure 4.4) shows that a little amount of SUIIc3 cox2 is expressed as insoluble protein and after cell lysis it is found in the pellet. Because the purification results with the standard protocol are not very satisfying (see Chapter 4.3.1), production of SUIIc3 cox2 in inclusion bodies could be a solution to obtain purified protein.

The denaturating step to solubilise inclusion bodies is already needed to unfold the protein for copper reconstitution of SUIIc3 cox2 (see Chapter 3.3.1.3.2) and it could be a way to achieve higher yields of reconstituted protein.

4.4.1 Expression of inclusion bodies

To promote the expression of inclusion bodies, after induction with IPTG the cells were grown in stress conditions at 42 °C.

Figure 4.20 shows the difference between expression of the protein at 37, 40 and 42 °C. Raising the temperature, more protein is found in the pellet than in the supernatant.

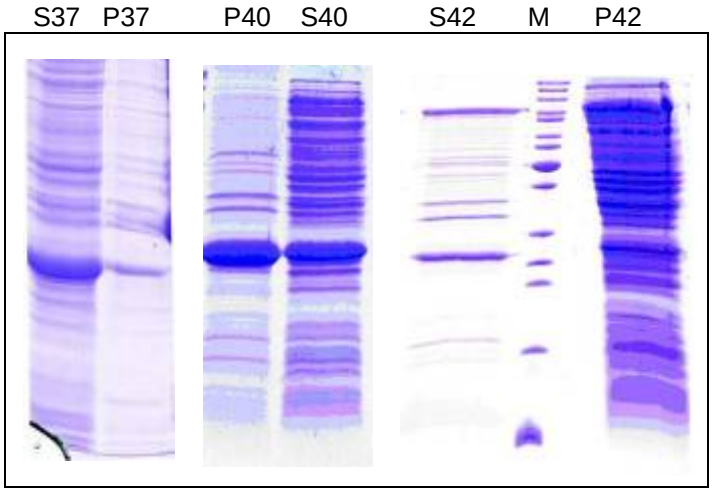


Figure 4.20: *SDS-PAGE of expression screenings at different expression temperatures. Lane M: marker; lane S37, 40, 42: supernatant from expression at 37, 40, 42 °C; lane P37, 40, 42: pellet from expression at 37, 40, 42 °C.*

4.4.2 Purification and solubilisation of inclusion bodies

Inclusion bodies were purified with several purification steps (see Chapter 3.4.2) and then solubilised with 6 M urea in 50 mM phosphate buffer, pH 6.5.

From each step a sample was taken from the supernatant and analysed by SDS-PAGE (Figure 4.21). As expected only a little amount of protein was in the supernatant.

Solubilisation also worked well: a large amount of protein could be solubilised, even if much more could be solubilised increasing the volume of the urea solution (see Figure 4.21).

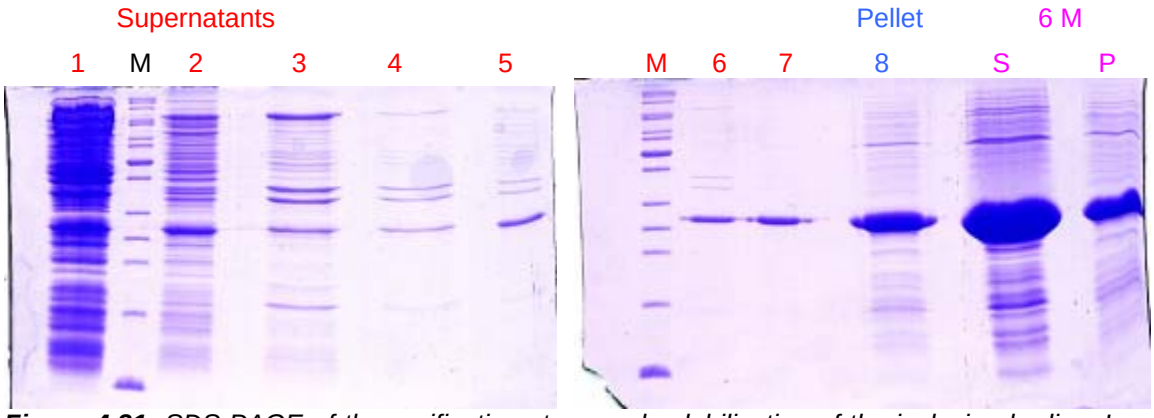


Figure 4.21: *SDS-PAGE of the purification steps and solubilisation of the inclusion bodies. Lane 1-7 (red): purification steps; lane 8 (blue): pellet before solubilisation; lane S (pink): solubilised inclusion bodies; lane P: pellet remained after solubilisation.*

4.4.3 Dialysis and copper reconstitution

To remove urea and reconstitute SUIIc3 cox2 the protein solution with 6 M urea was first dialysed overnight at 4°C in 50 mM phosphate buffer, pH 6.5, and 4 M urea with a protein solution to buffer ratio of 1:50. The buffer was then exchanged with a 50 mM phosphate buffer, pH 6.5, and 1 M solid urea and protein solution to buffer ratio of 1:50. The solution was dialysed for 3 hours at 4°C. The buffer was again exchanged with a 50 mM phosphate buffer, pH 6.5, without urea and protein solution to buffer ratio of 1:50. The solution was dialysed for 3 hours at 4°C. The buffer was exchanged for the last time with a 50 mM phosphate buffer, pH 6.5, with 1.5 mM $Cu(His)_2$ and protein solution to buffer ratio of 1:50. After overnight dialysis at 4°C the solution was slightly purple but a lot of protein was denaturated and precipitated.

The reconstituted SUIIc3 cox2 solution was concentrated with Amicon Centripreps. During concentration the $Cu(His)_2$ solution was washed out and the solution became more and more purple. The recorded UV-VIS spectrum (Figure 4.22) showed the typical absorbance maxima at about 480 nm and 535 nm. Only 30% (7 mg) of total SUIIc3 cox2 (21 mg) could be reconstituted, but analysis with SDS-PAGE (Figure 4.23) showed that the protein solution was much purer than after chromatographic purification.

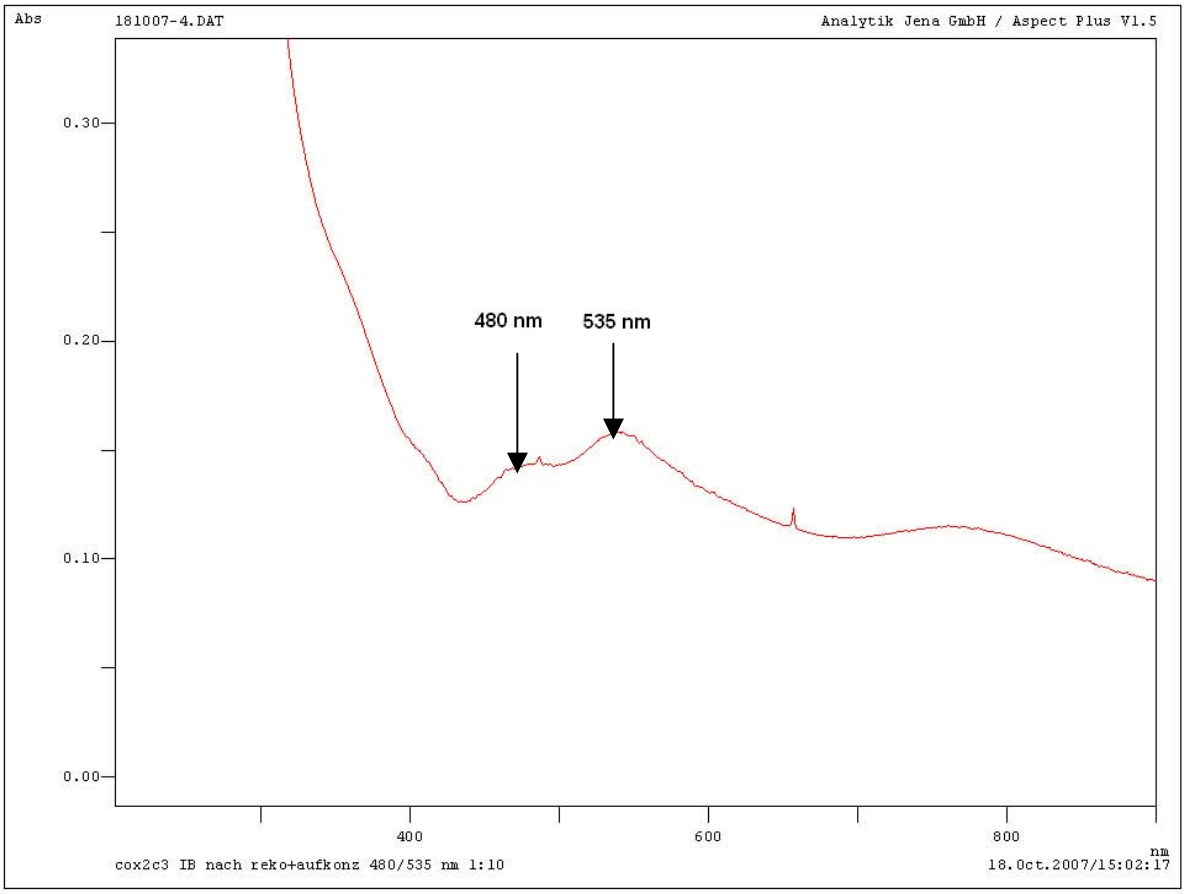


Figure 4.22: *UV-VIS spectrum of the reconstituted SUIIc3 cox2 with the inclusion bodie-protocol*

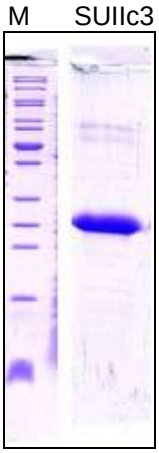


Figure 4.23: *SDS-PAGE of the reconstituted SUIIc3 cox2. Lane M: marker; lane SUIIc3: reconstituted SUIIc3 cox2*

4.5 Spectrophotometric investigations

4.5.1 Spectra of the recombinant proteins

4.5.1.1 Spectrum of the reconstituted, oxidised SUIIc3 cox2

The reconstituted, oxidised Cu_A-domain exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands. For a typical spectrum of the reconstituted SUIIc3 cox2 see Figure 4.17 or 4.22.

4.5.1.2 Spectrum of oxidised and reduced cytochrome c_M

Recombinat cytochrome c_M from *Nostoc sp.* PCC 7120 exhibited a typical heme absorption spectrum. In the completely reduced state three absorbance maxima could be observed: at 550 nm (α-peak), 521 nm (β-peak), and at 416 nm (γ- or Soret peak). In the oxidised state the Soret peak shifted to 406 nm, whereas the α- and β-peak converted into one broad peak with a maximum at 525 nm, as shown in Figure 4.24.

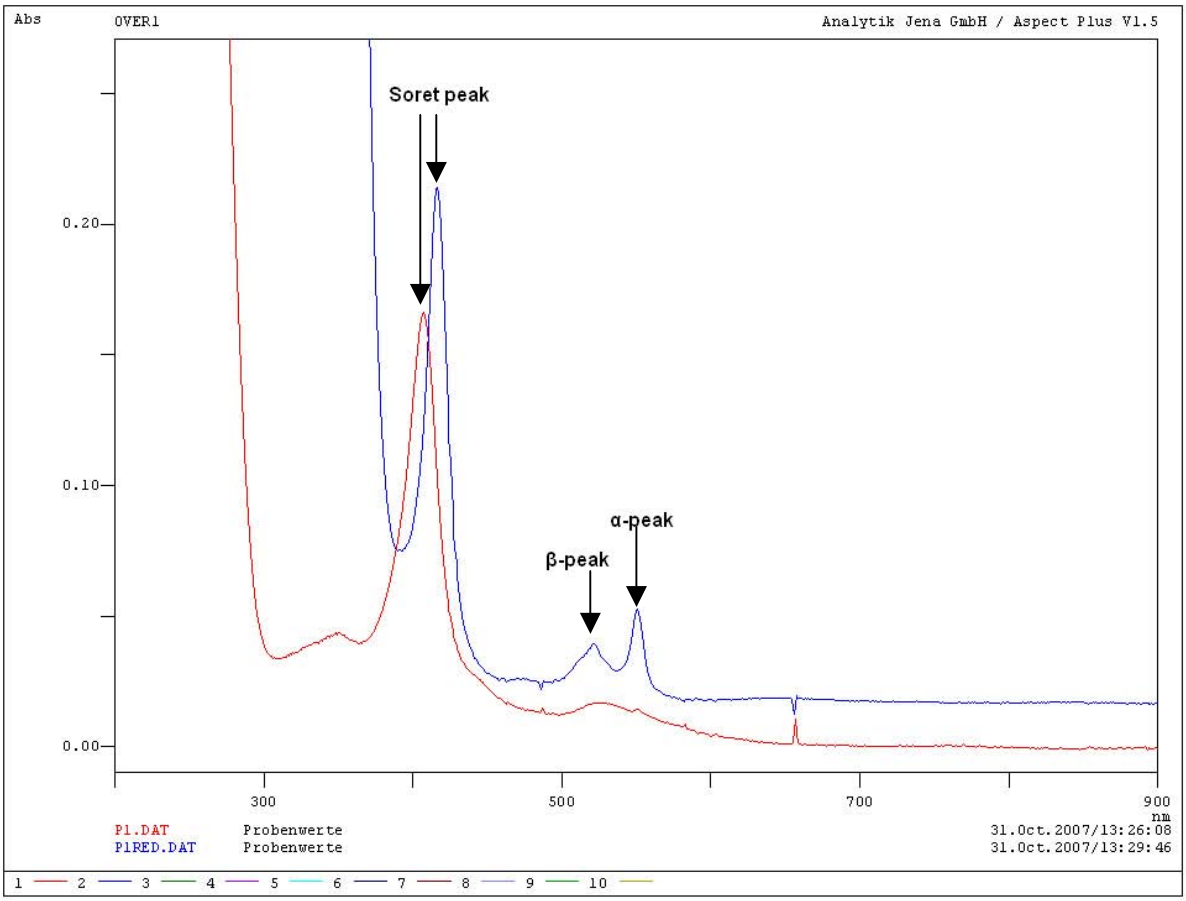


Figure 4.24: *Spectrum of cytochrome c_M in reduced (blue) and oxidized state (red) in 10 mM Tris/HCl buffer pH 8*

4.5.2 Determination of the molar extinction coefficient $\varepsilon_{280\ nm}$

The determined extinction coefficients of SUIIc3 cox2 and cytochrome c_M, calculated as described in Chapter 3.5.1 are summarised in Table 4.1

Table 4.1: Extinction coefficient at 280 nm

Protein	**$\varepsilon_{280\ nm}$**
SUIIc3 cox2	21.0 mM^{-1} cm^{-1}
cytochrome c_M	17.6 mM^{-1} cm^{-1}

4.5.3 Determination of protein concentration

4.5.3.1 Determination of SUIIc3 cox2 protein concentration

The protein concentration was calculated according to Lambert Beer's law as $c = A / \varepsilon d$ using ε_{280} = 21.0 mM^{-1} cm^{-1} for total protein concentration and ε_{480} = 2.8 mM^{-1} cm^{-1} or ε_{535} = 3.1 mM^{-1} cm^{-1} for the holoprotein concentration. Copper reconstitution efficiency was calculated as (mg total protein/mg holoprotein) x 100.
The results of the copper reconstitution with dialysis is shown in Table 4.2 and the results of the inclusion bodies experiment are given in Table 4.3.

Wavelength [nm]	ε [mM^{-1} cm^{-1}]	Concentration [mM]	Total protein / L *E.coli* culture [mg]	Copper reconstitution efficiency [%]
280	21.0	2.64	16.6 (8.3)*	10% (20)*
484	2.8	0.30	1.88	
540	3.1	0.28	1.75	

Table 4.2: *Concentration and yield of the copper reconstitution after dialysis and purification with Superdex™ 75*
*The solution still contained contaminant proteins: it was supposed that 50% of protein was SUIIc3 cox2

Wavelength [nm]	ε [mM^{-1} cm^{-1}]	Concentration [mM]	Total protein / L *E.coli* culture [mg]	Copper reconstitution efficiency [%]
280	21.0	1.73	23.28	31%
484	2.8	0.54	7.27	
540	3.1	0.54	7.27	

Table 4.3: *Concentration and yield of the copper reconstitution with the inclusion bodies-protocol.*

4.5.3.2 Determination of cytochrome c_M protein concentration

The protein concentration was calculated according to Lambert Beer's law as $c = A / \varepsilon d$ using ε_{280} = 17.6 mM^{-1} cm^{-1}. An average of 1 mg protein could be produced.

4.5.4 Reduction of cytochrome c_M

Unlike cytochrome c_M from *Synechocystis* PCC 6803, cytochrome c_M from *Nostoc* sp. PCC 7120 [109] is almost completely oxidised after cell lysis and oxidises completely during the purification procedure. The reduced protein seems to be

extremely unstable. So far, it was not possible to determine the molar extinction coefficient for 550-580 nm and 550-535 nm.

The capability of reduction was tested with a strong reduction agent (sodium-dithionite) and a weaker one (sodium-ascorbate). Sodium-dithionite could reduce cytochrome c_M (see Figure 4.24) but sodium-ascorbate could not, even if present in large excess (see Figure 4.25).

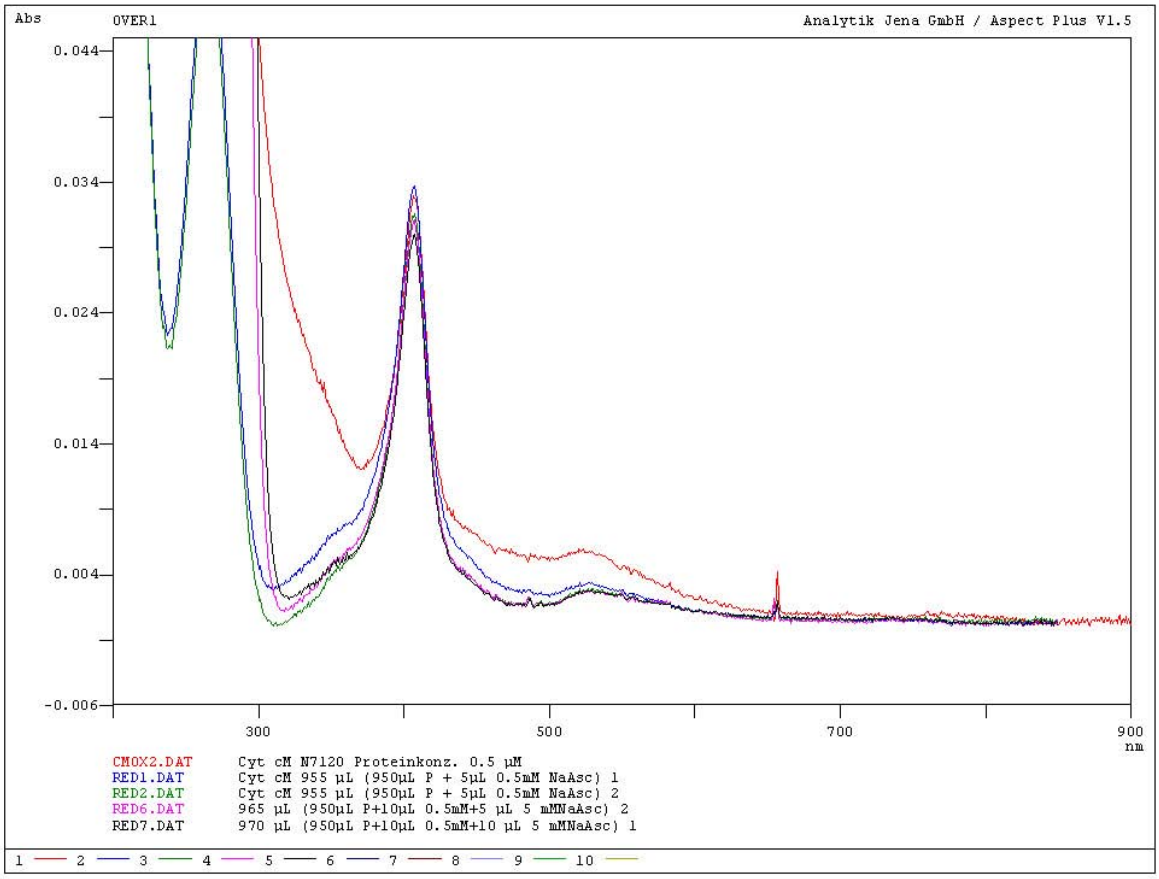


Figure 4.25: *Addition of large excess of sodium-ascorbate to reduce cytochrome c_M*

5. Discussion

5.1 Cloning of SUIIc3 cox2 and cytochrome c_M

E. coli BL21(DE3)pLysS were successfully transformed with the pET-3a plasmid carrying the cytochrome *c* oxidase 2 subunit II insert (amino acid residues 113-327). The protein has the same cutting site as SUIId2 cox1 (amino acid residues 113-355), comprising both the electron donor binding site and the electron entry site but without the two transmembrane helices at the N-terminus of subunit II.
The 655 bp long PCR-product was digested with *Bam*HI and *Nde*I, ligated with the pET-3a vector and transferred in *E. coli* BL21(DE3)pLysS. This expression system was already used by Judith Schachinger for SUIIj3 cox2 (amino acid residues 137-327) [110]. PCR-screening showed that clone c3 was carrying the insert at the right length. SDS-PAGE after expression screening confirmed that the clone was also expressing a good amount of soluble protein with 22.5 kDa molecular weight, which corresponds to the calculated molecular weight of SUIIc3 cox2. Sequencing of DNA purified from the transformed cells proofed that it contained no mutations that lead to differences in the proteins' amino acid sequence.

The heme protein cytochrome c_M (without the putative signal sequence) was also successfully cloned in *E. coli* BL21(DE3)Star using the pET-27b+ plasmid. The cells were already carrying the pEC86 plasmid which encodes the cytochrome *c* maturation genes.
The insert coding for cytochrome c_M was 231 bp long. After amplification by PCR, it was digested with *Bam*HI and *Nco*I, ligated with the pET-27b+ vector and transferred in *E. coli* BL21(DE3)Star. The same vector and cells worked well to recombinantly produce and overexpress cytochrome c_6 from *Synechocystis* sp. PCC 6803 [107, 108]. Three positive clones were found after PCR-screening. All clones were also expressing protein with 8.3 kDA molecular weight, even if SDS-PAGE showed that a high amount of protein was not in soluble form. Spectra of the supernatant after cell lysis showed that clone c1 was expressing the highest amount of soluble protein. Sequencing of DNA confirmed finally that it contained no mutations that lead to differences in the proteins' amino acid sequence.

5.2 Expression and purification of SUIIc3 and cytochrome c_M

The SUIIc3 cox2 was expressed in the *E. coli* strain BL21(DE3)pLysS using a protocol similar to that of SUIIj3 cox2 [110], but with a longer expression time (20 h) at low temperature (16 °C) to promote the correct folding of the protein. In order to obtain a pure, functional holoprotein, two chromatographic steps, comprising Q-sepharose and Superdex^{TM} 75, were performed and between them it was tried to reconstitute SUIIc3 cox2 with two approaches. The protocol used for copper reconstitution in *Synechocystis* sp. PCC 6803 did not work. Reconstitution after unfolding with urea followed by dialysis was successful but the amount of reconstituted SUIIc3 cox2 was only 20% of the total protein amount (an average of 10 mg per litre culture volume), not enough for kinetic measurements, and SDS-PAGE showed that the protein solution is not pure.
Higher yields of purified, functional holoprotein were obtained by production of SUIIc3 cox2 as inclusion bodies followed by solubilisation and reconstitution: 20 mg total protein could be obtained per litre culture volume with about 30% holoprotein of high purity. Even if these results are better than that obtained with the standard protocol, they are far away to be statisfying because of the low copper occupancy.

In order to express cytochrome c_M in amount high enough to perform kinetic measurements an expression protocol had to be established. Tests on culture conditions showed that the best results were obtainable using M9ZB$_{KanCm}$ medium, a reduction of the incubation temperature from 37°C to 30°C and addition of 1 mM IPTG at a suitable cell density. The optimal culture duration was determined to be 10 hours at 180 rpm. Hemin which was supposed to support heme insertion did not lead to an increase of produced cytochrome c_M.
With the three-step purification procedure already used for cytochrome c_M from *Synechocystis* sp. PCC 6803 [109], comprising an anion exchange chromatography with DEAE-sepharose, a cation exchange chromatography with CM-sepharose and a size exclusion chromatography with Superdex^{TM} 75, a yield of 1 mg cytochrome c_M per litre culture volume could be obtained. This amount is comparable with the 1.5 mg obtained for cytochrome c_M from *Synechocystis* sp. PCC 6803. [109]

5.3 Spectrophotometric characterisation of SUIIc3 and cytochrome c_M

As expected, the reconstituted, oxidised Cu_A-domain exhibits two strong absorbance maxima at about 480 nm and 535 nm, arising from the interaction of the two copper ions with each other and with the ligands.

UV-VIS absorption spectra of cytochrome c_M from *Nostoc sp.* PCC 7120 showed a typical heme absorption spectrum. In the completely reduced state three absorbance maxima could be observed: at 550 nm (α-peak), 521 nm (β-peak), and at 416 nm (γ- or Soret peak). In the oxidised state the Soret peak shifted to 406 nm, whereas the α- and β- peak converted into one broad peak with a maximum at 525 nm. The maxima were found at the same wavelength as cytochrome c_M from *Synechocystis* sp. PCC 6803 [109].

The molar extinction coefficients at 280 mm of both SUIIc3 cox2 and cytochrome c_M, were determined and are shown in Table 5.1.

Table 5.1: *Extinction coefficient at 280 nm*

Protein	**$\varepsilon_{280\ nm}$**
SUIIc3 cox2	21.0 mM^{-1} cm^{-1}
cytochrome c_M	17.6 mM^{-1} cm^{-1}

Unlike cytochrome c_M from *Synechocystis* PCC 6803, cytochrome c_M from *Nostoc* sp. PCC 7120 [109] is almost completely oxidised after cell lysis and oxidises completely during the purification procedure. Only addition of a strong reduction agent as sodium-dithionite can reduce the protein. Even a large excess of sodium-ascorbate, a weaker reduction agent, could not reduce the protein. It seems that cytochrome c_M from *Nostoc* sp. PCC 7120 is even more unstable in the reduced state as cytochrome c_M from *Synechocystis* PCC 6803.

So far, no kinetic measurements with SUIIc3 cox2 and cytochrome c_M could be performed. Higher yields of redox-active Cu_A domain and cytochrome c_M have to be achieved, as well as a more stable, reduced cytochrome c_M to study the physiological

role of this protein and its putative role as electron donor of cytochrome oxidase under stress conditions.

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